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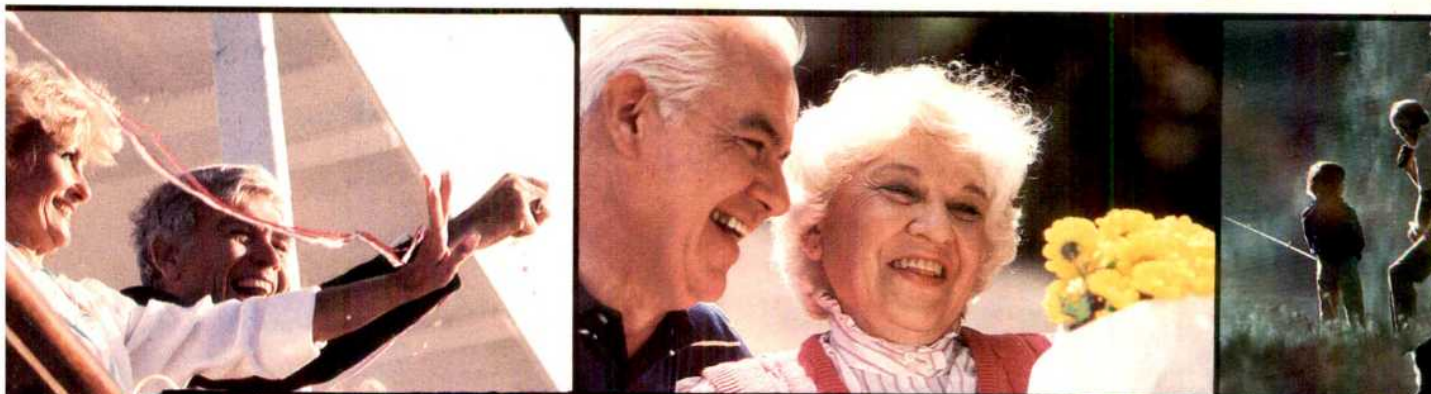
*American Society of Hematology
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ABSTRACT DEADLINE: SEPTEMBER 1, 1987

Table of Contents v

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¹Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.



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Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g., methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below $5 \times 10^{-8} M$.^{1,2}

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e., trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

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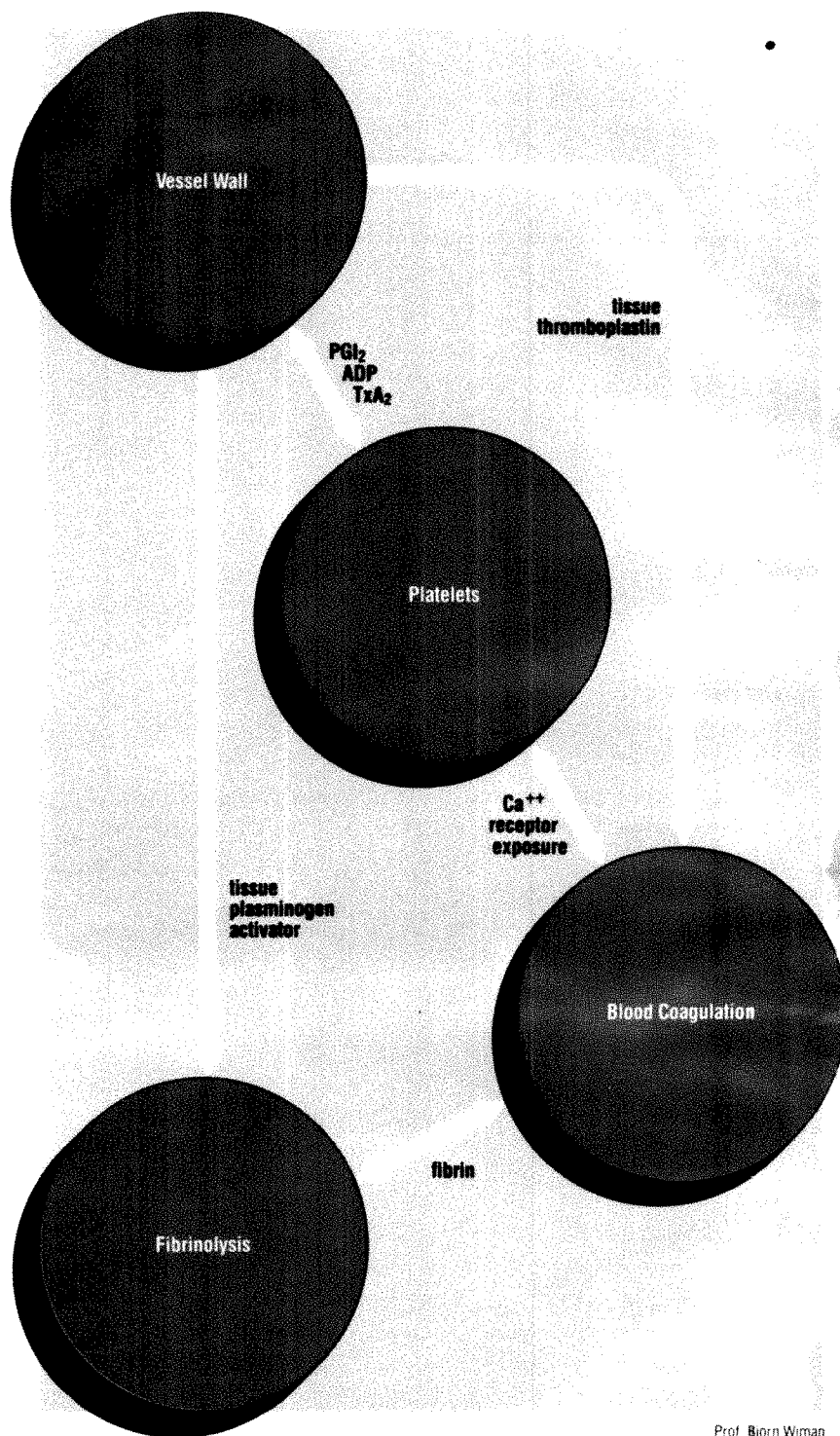
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*The Journal of
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VOL 70, NO 1

JULY 1987

CONTENTS

Review: The Coagulation-Kinin Pathway of Human Plasma	<i>Allen P. Kaplan and Michael Silverberg</i>	1
A Human Monoclonal Autoantibody Recognizes a Neoantigen on Glycoprotein IIIA Expressed on Stored and Activated Platelets.....	<i>Diane J. Nugent, Thomas J. Kunicki, Cicely Berglund, and Irwin D. Bernstein</i>	16
An Evaluation of Crossmatching, HLA, and ABO Matching for Platelet Transfusions to Refractory Patients	<i>Joanna M. Heal, Neil Blumberg, and Debra Masel</i>	23
Identification of a Defective Factor XI Cross-Reacting Material in a Factor XI-Deficient Patient.....	<i>C. Mannhalter, P. Hellstern, and E. Deutsch</i>	31
Anemia of the Belgrade Rat: Evidence for Defective Membrane Transport of Iron.....	<i>Barbara J. Bowen and Evan H. Morgan</i>	38
Analysis With Antiidiotype Antibody of a Patient With Chronic Lymphocytic Leukemia and a Large Cell Lymphoma (Richter's Syndrome).....	<i>L.F. Bertoli, H. Kubagawa, G.V. Borzillo, M. Mayumi, J.T. Prechal, J.F. Kearney, J.R. Durant, and M.D. Cooper</i>	45
Clinical Significance of Low Levels of Myeloperoxidase Positivity in Childhood Acute Nonlymphoblastic Leukemia	<i>Ching-Hon Pui, Frederick G. Behm, David K. Kalwinsky, Sharon B. Murphy, Debra L. Butler, Gary V. Dahl, and Joseph Mirro</i>	51
Recombinant Human TNF _α Stimulates Production of Granulocyte Colony-Stimulating Factor	<i>H. Phillip Koefler, Judith Gasson, John Ranyard, Larry Souza, Michael Shepard, and Reinhold Munker</i>	55
Changes in Neutrophil Surface Protein Composition Accompany Phagocytosis	<i>Keith M. Skubitz and Tracy K. Kinkead</i>	60
Immunological Specificity and Mechanism of Action of IgG Lupus Anticoagulants	<i>Vittorio Pengo, Perumal Thiagarajan, Sandor S. Shapiro, and Marilyn J. Heine</i>	69
Cytoplasmic Factor Required for Entry of Malaria Parasites Into RBCs.....	<i>K. Rangachari, A.R. Dluzewski, R.J.M. Wilson, and W.B. Gratzer</i>	77
Immunophenotyping of Acute Myeloid Leukemia Using Monoclonal Antibodies and the Alkaline Phosphatase-Antialkaline Phosphatase Technique.....	<i>Curtis A. Hanson, Kazimiera J. Gajl-Peczalska, Janet L. Parkin, and Richard D. Brunning</i>	83
The Gene Located at Chromosome 18 Band q21 Is Rearranged in Uncultured Diffuse Lymphomas as Well as Follicular Lymphomas.....	<i>Ming-Sheng Lee, Mark B. Blick, Sen Pathak, Jose M. Trujillo, James J. Butler, Ruth L. Katz, Peter McLaughlin, Frederick B. Hagemeister, William S. Velasquez, Angela Goodacre, Ann Cork, Jordan U. Gutterman, and Fernando Cabanillas</i>	90
Rearranged Antigen Receptor Genes in Hodgkin's Disease	<i>James Sundeen, Edward Lipford, Michael Uppenkamp, Eileen Sussman, Larry Wahl, Mark Raffeld, and Jeffrey Cossman</i>	96
Expression on Blood Cells of Sialophorin, the Surface Glycoprotein That Is Defective in Wiskott-Aldrich Syndrome	<i>Eileen Remold-O'Donnell, Christopher Zimmerman, Dianne Kenney, and Fred S. Rosen</i>	104
Arginyl-Glycyl-Aspartic Acid Sequences and Fibrinogen Binding to Platelets	<i>Edward F. Plow, Michael D. Pierschbacher, Erkki Ruoslahti, Gerard Marguerie, and Mark H. Ginsberg</i>	110

(Continued on following page)

(Contents continued)

Second Marrow Transplants in Patients With Aplastic Anemia Rejecting the First Graft: Use of a Conditioning Regimen Including Cyclophosphamide and Antithymocyte Globulin	<i>R. Storb, P.L. Weiden, K.M. Sullivan, F.R. Appelbaum, P. Beatty, C.D. Buckner, R.A. Clift, K.C. Doney, J. Hansen, P.J. Martin, J.E. Sanders, P. Stewart, R.P. Witherspoon, and E.D. Thomas</i>	116
Loss of Myeloid Differentiation Antigens Precedes Blastic Transformation in Chronic Myelogenous Leukemia	<i>Mary B. Todd, James A. Waldron, Timothy A. Jennings, Lisa S. Rome, Sanford D. Markowitz, Theodore R. Holford, Joseph P. Gardner, John P. Wolak, and Harry L. Malech</i>	122
Low Molecular Weight B Cell Growth Factor Induces Proliferation of Human B Cell Precursor Acute Lymphoblastic Leukemias.....	<i>Bernhard Wörmann, Shashikant R. Mehta, Abby L. Maizel, and Tucker W. LeBien</i>	132
Epitope Mapping of Functional Domains of Human Factor Va With Human and Murine Monoclonal Antibodies. Evidence for the Interaction of Heavy Chain With Factor Xa and Calcium.....	<i>Anjanayaki E. Annamalai, A. Koneti Rao, Hui Chong Chiu, Danny Wang, Asim K. Dutta-Roy, Peter N. Walsh, and Robert W. Colman</i>	139
A New Mutation in IVS-1 of the Human β Globin Gene Causing β Thalassemia Due to Abnormal Splicing	<i>George F. Atweh, Corinne Wong, Robin Reed, Stylianos E. Antonarakis, Ding-er Zhu, Prahbat K. Ghosh, Tom Maniatis, Bernard G. Forget, and Haig H. Kazazian, Jr</i>	147
Endothelial Cell Interactions With Sick Cell, Sick Trait, Mechanically Injured, and Normal Erythrocytes Under Controlled Flow	<i>Gilda A. Barabino, Larry V. McIntire, Suzanne G. Eskin, David A. Sears, and Mark Udden</i>	152
Erythrocyte Membrane Skeleton Abnormalities in Severe β -Thalassemia	<i>E. Shinar, O. Shalev, E.A. Rachmilewitz, and S.L. Schrier</i>	158
Development of the Human Coagulation System in the Full-Term Infant	<i>Maureen Andrew, Bosco Paes, Ruth Milner, Marilyn Johnston, Lesley Mitchell, Douglas M. Tollefsen, and Peter Powers</i>	165
Proteolytic Degradation of von Willebrand Factor After DDAVP Administration in Normal Individuals	<i>Javier Batlle, Maria Fernanda Lopez-Fernandez, A. Lopez-Borrascas, Consuelo Lopez-Berges, Judith A. Dent, Scott D. Berkowitz, Zaverio M. Ruggeri, and Theodore S. Zimmerman</i>	173
Regulation of Thrombopoiesis: Effects of the Degree of Thrombocytopenia on Megakaryocyte Ploidy and Platelet Volume.....	<i>Laurence Corash, Hui Yin Chen, Jack Levin, Georgiann Baker, Helen Lu, and Yuen Mok</i>	177
Clonal Immunoglobulin Gene Rearrangements in Tissues Involved by Hodgkin's Disease.....	<i>Marja G.L. Brinker, Sibrand Poppema, Charles H.C.M. Buys, Wim Timens, Jan Osinga, and Lydia Visser</i>	186
Growth Factor Requirements of Childhood Acute Leukemia: Establishment of GM-CSF-Dependent Cell Lines	<i>Beverly Lange, Mauro Valtieri, Daniela Santoli, Daniele Caracciolo, Fulvio Mavilio, Ingrid Gemperlein, Constance Griffin, Beverly Emanuel, Janet Finan, Peter Nowell, and Giovanni Rovera</i>	192
Recombinant Human Tumor Necrosis Factor $_{\alpha}$ Regulates <i>c-myc</i> Expression in HL-60 Cells at the Level of Transcription	<i>A. Tobler, D. Johnston, and H.P. Koeffler</i>	200
Recombinant Human GM-CSF Induces Leukocytosis and Activates Peripheral Blood Polymorphonuclear Neutrophils in Nonhuman Primates	<i>P. Mayer, C. Lam, H. Obenaus, E. Liehl, and J. Besemer</i>	206
S-100 Beta Positive Human T Lymphocytes: Their Characteristics and Behavior Under Normal and Pathologic Conditions	<i>Kiyoshi Takahashi, Tadashi Yoshino, Kazuhiko Hayashi, Hiroshi Sonobe, and Yuji Ohtsuki</i>	214
Fibrinogen-Independent Aggregation and Deaggregation of Human Platelets: Studies in Two Afibrinogenemic Patients	<i>M. Cattaneo, R.L. Kinlough-Rathbone, A. Lecchi, C. Bevilacqua, M.A. Packham, and J.F. Mustard</i>	221
HLA Associations With Leukemia.....	<i>Mortimer M. Bortin, Joseph D'Amato, Fritz H. Bach, Alfred A. Rimm, and Jon J. van Rood</i>	227
Identification of Molecular Variants of p210 ^{bcr-abl} in Chronic Myelogenous Leukemia	<i>Razelle Kurzrock, William S. Kloetzer, Moshe Talpaz, Mark Blick, Ronald Walters, Ralph B. Arlinghaus, and Jordan U. Gutterman</i>	233

(Continued on following page)

(Contents continued)

Pseudosickling of Hemoglobin Setif	<i>Samuel Charache, Eva Raik, David Holtzclaw, Patricia J. Hathaway, Elizabeth Powell, and Patricia Fleming</i>	237
Clinical Experience With Transfusion of Leukocyte-Poor Platelet Concentrates Prepared by Filtration With Prostacyclin.....	<i>Hendrik C. van Prooijen, Tineke I. Riemens, and Jan Willem N. Akkerman</i>	243
Hypodiploidy Is Associated With a Poor Prognosis in Childhood Acute Lymphoblastic Leukemia	<i>Ching-Hon Pui, Dorothy L. Williams, Susana C. Raimondi, Gaston K. Rivera, A. Thomas Look, Richard K. Dodge, Stephen L. George, Frederick G. Behm, William M. Crist, and Sharon B. Murphy</i>	247
Effects of B Cell Stimulatory Factor-1/Interleukin 4 on Hematopoietic Progenitor Cells	<i>Christian Peschel, William E. Paul, Junichi Ohara, and Ira Green</i>	254
Lactoferrin Binding by Leukemia Cell Lines	<i>Yasuaki Yamada, Tatsuhiko Amagasaki, Donald W. Jacobsen, and Ralph Green</i>	264
CFU-GM Content of Bone Marrow Graft Correlates With Time to Hematologic Reconstitution Following Autologous Bone Marrow Transplantation With 4-Hydroperoxycyclophosphamide-Purged Bone Marrow	<i>Scott D. Rowley, Michael Zuehlendorf, Hayden G. Braine, O. Michael Colvin, Janice Davis, Richard J. Jones, Rein Saral, Lyle L. Sensenbrenner, Andrew Yeager, and George W. Santos</i>	271
Immunologic Aberrations, HIV Seropositivity and Seroconversion Rates in Patients With Hemophilia B	<i>Doreen B. Brettler, Frank Brewster, Peter H. Levine, Ann Forsberg, Sharon Baker, and John L. Sullivan</i>	276
Plasma and Platelet HLA in Normal Individuals: Quantitation by Competitive Enzyme-Linked Immunoassay	<i>K.J. Kao</i>	282
Non-Hodgkin's Lymphoma Containing Both B and T Cell Clones	<i>Eddie Hu, Lawrence M. Weiss, Roger Warnke, and Jeffrey Sklar</i>	287
Equal Stabilities of Normal β Globin and Nontranslatable β^0 -39 Thalassemic Transcripts in Cell-Free Extracts	<i>C.A. Stolle, M.S. Payne, and E.J. Benz, Jr</i>	293
Biosynthesis and Secretion of Functional Protein S by a Human Megakaryoblastic Cell Line (MEG-01)	<i>Michinori Ogura, Noboru Tanabe, Junji Nishioka, Koji Suzuki, and Hidehiko Saito</i>	301
Detection of Activated Platelets in Whole Blood Using Activation-Dependent Monoclonal Antibodies and Flow Cytometry	<i>Sanford J. Shattill, Michael Cunningham, and James A. Hoxie</i>	307

CONCISE REPORTS

Physiologic Regulation and Tissue Localization of Renal Erythropoietin Messenger RNA	<i>Stephen J. Schuster, Jean H. Wilson, Allan J. Erslev, and Jaime Caro</i>	316
The Effect of the Plasticizer Di(2-ethylhexyl)phthalate on Red Cell Deformability	<i>R.S. Labow, R.T. Card, and G. Rock</i>	319
Marrow Transplantation for Children With Acute Lymphoblastic Leukemia in Second Remission	<i>Jean E. Sanders, E. Donnell Thomas, C. Dean Buckner, and Kristine Doney</i>	324
Identification of a Restriction Fragment Length Polymorphism Involving the Oncogene ETS-1 on Chromosome 11q23.....	<i>Paul D. Savage, Curtis A. Hanson, and John H. Kersey</i>	327
Localization of the G-CSF Gene on Chromosome 17 Proximal to the Breakpoint in the t(15;17) in Acute Promyelocytic Leukemia	<i>Ruth N. Simmers, Lorna M. Webber, M. Frances Shannon, O. Margaret Garson, Gordon Wong, Mathew A. Vadas, and Grant R. Sutherland</i>	330
Human Recombinant Multilineage Colony Stimulating Factor (Interleukin-3): Stimulator of Acute Myelocytic Leukemia Progenitor Cells In Vitro	<i>Ruud Delwel, Lambert Dorssers, Ivo Touw, Gerard Wagemaker, and Bob Löwenberg</i>	333

(Continued on following page)

(Contents continued)

SPECIAL DEPARTMENTS

Correspondence	337
Errata	339
Announcements	340
Information for Contributors	xxxv, xxxvi

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A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of the lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O₂ at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban[®]. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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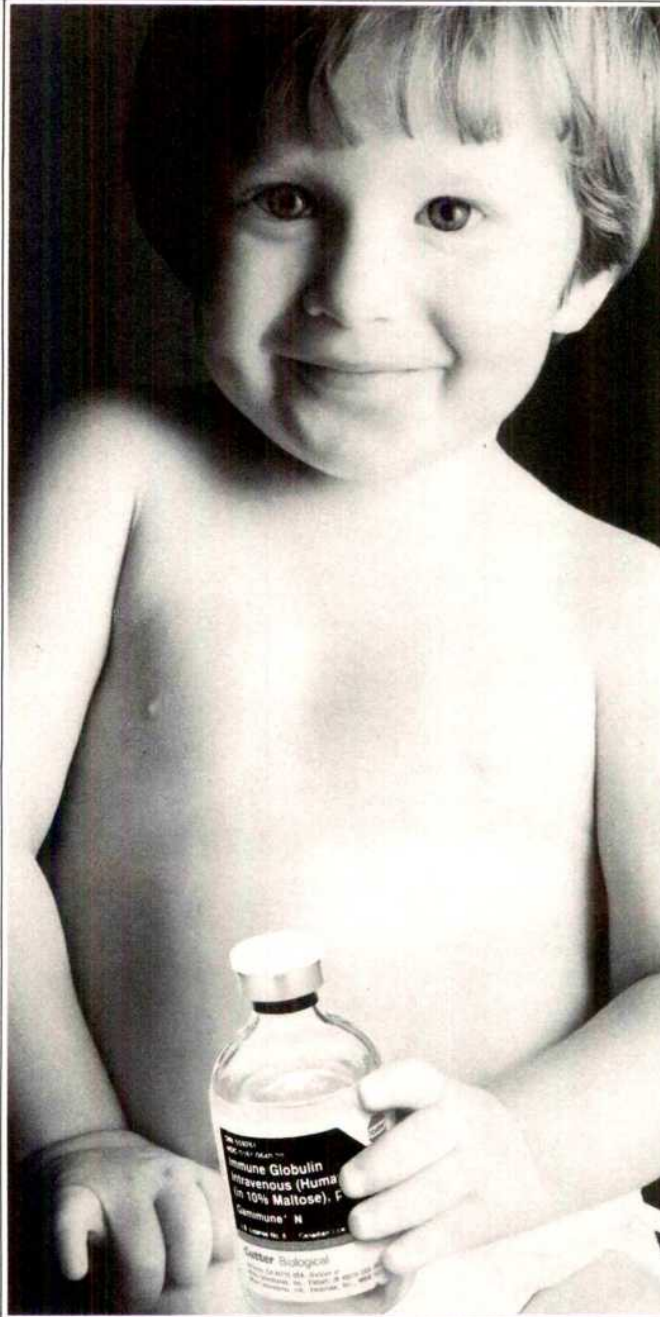
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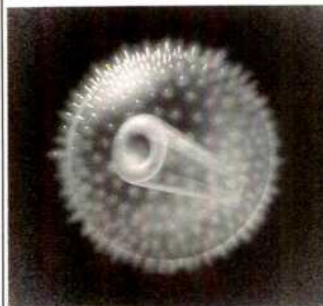


Documented in vitro inactivation of HTLV-III virus[‡]

[‡]The Cohn-Oncley process used to produce Gamimune®N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HTLV-III and found to remove and/or inactivate the viral challenge. In addition, Gamimune®N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 TCID (Tissue Culture Infectious Doses) reduction of HTLV-III.³

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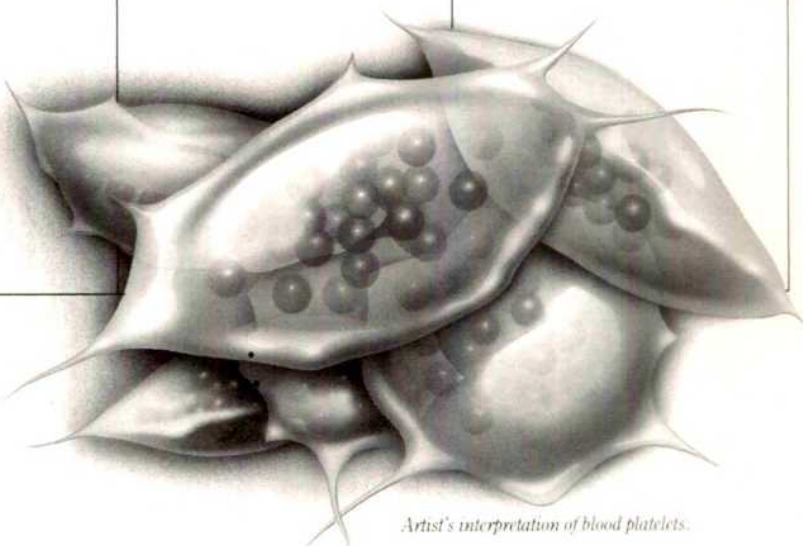
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Artist's interpretation of HTLV-III virus.

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Artist's interpretation of blood platelets.

*It is not possible to predict which patients with ITP will respond to therapy.

¹In patients who respond to therapy, a rise in platelet count is generally rapid (within 1 to 5 days), transient (most often lasting from several days to several weeks) and should not be considered curative.

From the original IGIV, today's IGIV...it offers more

Please see following page for brief summary of prescribing information.

GAMIMUNE® N

Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25

CLINICAL PHARMACOLOGY

Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood, there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~ 0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight, following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLA₁ alloimmune antibody with platelet anti-PLA₁ specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered, in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgia, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be **gradually** increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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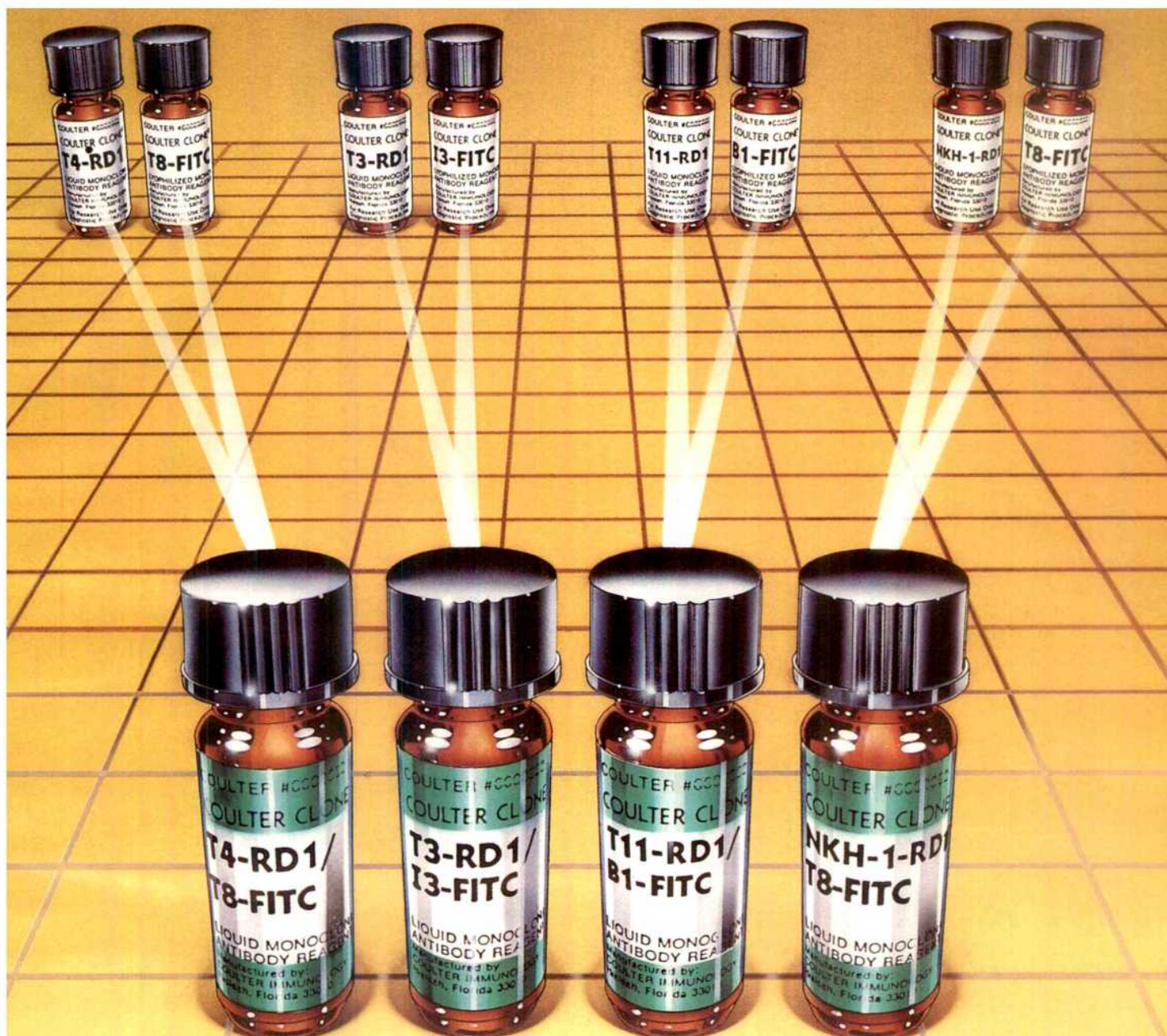
REFERENCES: 1. GAMIMUNE® N Product License Application. 2. Lundblad JL, Mitra G, Steinberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(Jul-Aug): S382-S390. 3. Mitra G, Wong MF, Mozen MM, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26(4):394-397.

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Radiogenic mucositis or herpes simplex?

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A high percentage of immunocompromised patients are at risk for developing HSV infections. Timely therapy with an antiviral agent can avoid the incidence of secondary complications, such as visceral dissemination, and prolonged hospital stay.

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The diagnostic
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Please see following page for brief summary of prescribing information.

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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M² every 8 hours (750 mg/M²/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.^{1,2,3}

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.⁴

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.⁵

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.⁶

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS AND DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency—4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 5 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used

in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and LS178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonia, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.3 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 AND HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS — 5 mg/kg infused at a constant rate over 1 hour every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, every 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

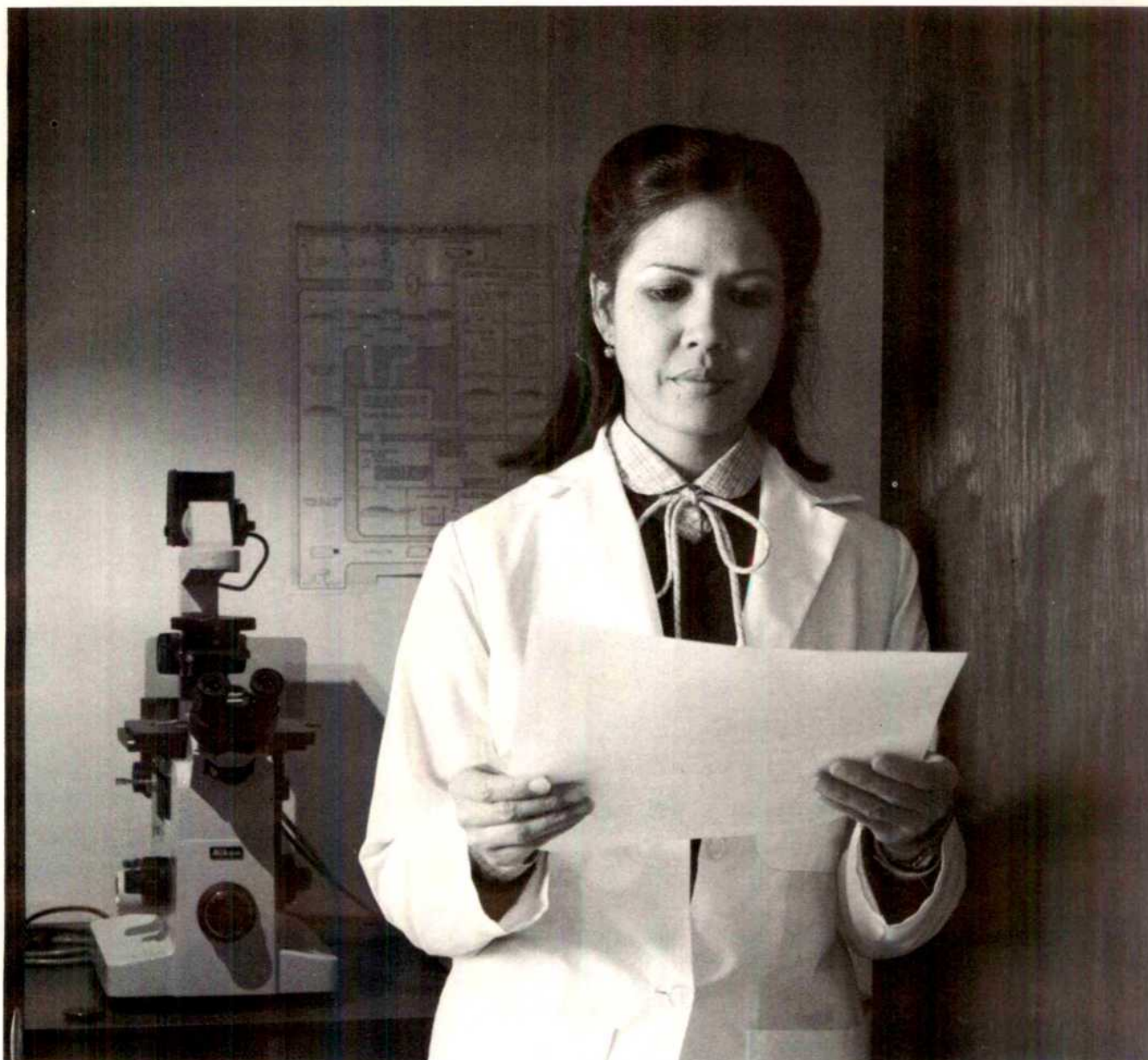
Creatinine Clearance (ml/min/1.73M ²)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, et al., *Lancet* 1(8235): 1389-1392, Jun. 27, 1981. 2. Wade, et al., *Ann. Intern. Med.* 96(3): 265-269, Mar. 1982. 3. J.D. Meyers, et al., *Am J. Med.* 73(1A): 229-235, Jul. 20, 1982. 4. Data on file, Burroughs Wellcome Co. 5. A. Mindel, et al., *Lancet* 1(8274): 697-700, Mar. 27, 1982. 6. Z.M. Naib, et al., *Cancer Res.* 33:1452-1463, 1973.

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*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

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Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches: elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. 2. Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. 3. Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. 4. Data on file, Miles Pharmaceuticals.



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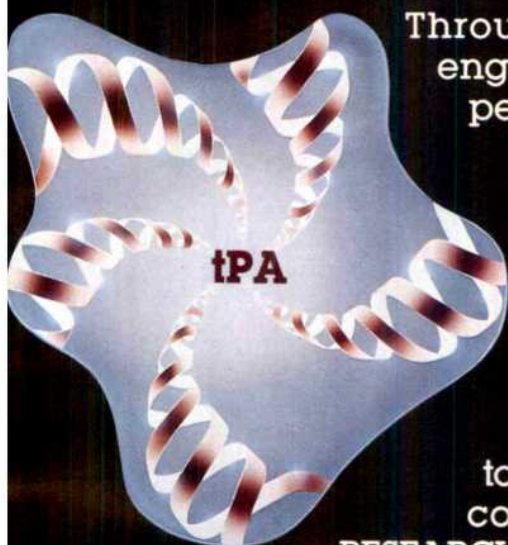


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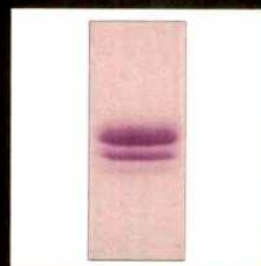
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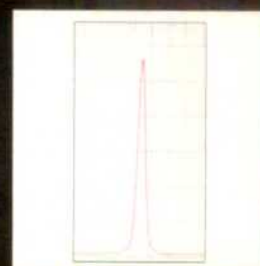
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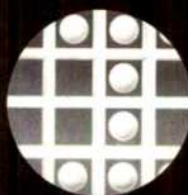
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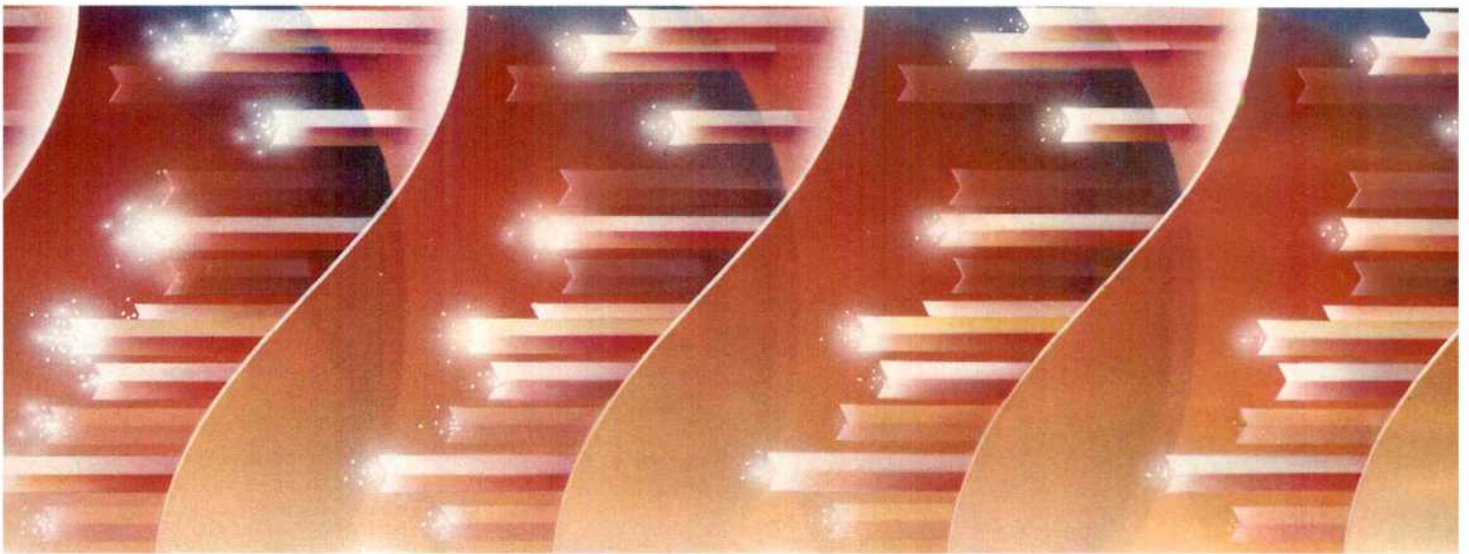
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REVIEW

The Coagulation-Kinin Pathway of Human Plasma

By Allen P. Kaplan and Michael Silverberg

INITIATION of the intrinsic coagulation-kinin pathway requires the interaction of certain negatively charged surfaces with three plasma proteins: factor XII (Hageman factor [HF]),¹ prekallikrein,^{2,4} and high-molecular weight (HMW) kininogen.⁵⁻⁷ Prekallikrein and HMW kininogen circulate in plasma as a complex with a 1:1 molar stoichiometry.⁸ These proteins bind to initiating surfaces, HF becomes activated, prekallikrein is converted to kallikrein, and HMW kininogen is digested to release the vasoactive peptide bradykinin (Fig 1). A second major plasma substrate of activated HF (HFa), factor XI, also circulates as a complex with HMW kininogen⁹ and is converted to factor XIa,¹⁰ which continues the intrinsic coagulation cascade. During the past 15 years, considerable progress has been made in elucidating the molecular details of these reactions. The aforementioned factors also interact with other plasma proteins, the peripheral vasculature, and with a variety of cell types to contribute to the body's inflammatory response. In addition, new methods have been developed to assess the contribution of this portion of the coagulation cascade to human disease. This review will include progress made in each of these areas, controversies that exist, and directions for future investigation.

INITIATION OF CONTACT ACTIVATION

Human HF, coagulation factor XII, is a single-chain β globulin of molecular weight 80,000.¹¹ During contact activation, HF becomes cleaved and then fragmented.^{12,13} The structural changes are accompanied by the development of enzymatic activity expressed against low-molecular weight (LMW) peptide derivatives as well as a number of plasma proteins. One paradox of contact activation is that kallikrein, the major plasma protease capable of activating HF¹⁴ (Fig 1) and other weaker activators (factor XIa and plasmin), are themselves formed in HF-dependent reactions.^{13,14} Purified HF and prekallikrein show minimal enzymatic activity if precautions are taken to eliminate contaminating active enzyme. However, when mixtures of HF and prekallikrein are exposed to an "activating" macromolecular anionic material (surface), rapid activation of both proteins takes place.

Any mechanism proposed for the initiation of contact activation must explain the behavior of normal and deficient plasmas. An important example is Fletcher trait plasma, which is deficient in prekallikrein^{2,4} and has a coagulation

defect that is corrected upon reconstitution with prekallikrein. If the time of incubation with surfaces is increased, the clotting time in a two-stage assay approaches that of normal plasma¹⁵⁻¹⁷; thus, surface-dependent activation of HF can occur in the absence of prekallikrein, and HFa can in turn convert factor XI to factor XIa so that coagulation can proceed. Little kinin is formed, however, because prekallikrein is absent (Fig 1). Small amounts of kinin may be made relatively slowly by direct cleavage of HMW kininogen by HFa¹⁸ or factor XIa, but the amounts are unlikely to be physiologically significant. In contrast, HF-deficient plasma does not activate upon contact with a surface. These observations draw attention to the central role of HF in contact activation; prekallikrein apparently plays an accelerating rather than a required role. This concept is supported by the discovery that human HF is able to autoactivate,^{19,20} a feature shared with the rabbit contact system²¹ but apparently not the bovine system.²² The autoactivation of HF may explain its activation in prekallikrein-deficient plasma and the consequent correction of the clotting defect.

The ability of HFa to cleave and activate native HF has been demonstrated directly for both rabbit²¹ and human proteins.^{20,23} In addition, the kinetics of the appearance of activity in purified HF exposed to a surface has been analyzed by an iterative computer model,²⁰ an explicit second-order kinetic model,²⁴ and by demonstration of exponential initial rates.^{25,26} All of these analyses have yielded data consistent with a simple autoactivation scheme in which HFa digests HF to produce more HFa.

The autoactivation of HF focuses our attention on the interaction of HF with a surface. This reaction poses the same dilemma as does the complete contact activation reaction in plasma; namely, how does one or more species of zymogen develop enzymatic activity when exposed to a

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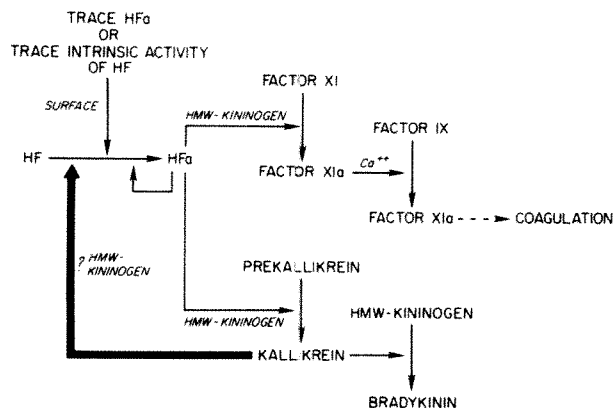


Fig 1. Schematic diagram of the contact activation pathway depicting HF autoactivation, activation of prekallikrein and factor XI, and the generation of bradykinin. The major cofactor role of HMW kininogen is on activation of HF substrates. Its direct role in HF activation by kallikrein is less clear and varies with the surface used and the milieu as described in the text.

charged but otherwise chemically inert surface. Hypotheses regarding the mechanism of initiation of contact activation may be divided into two categories. We can posit either that the surface amplifies traces of activity present in zymogen *ab initio* or that it functions in some manner to induce activity in previously inactive molecules. McMillin et al and Ratnoff and Saito originally proposed that HF undergoes a conformational change upon binding to a surface whereby it exposes or creates an active site that is then capable of activating prekallikrein.^{27,28} In this hypothesis, the induction of a low level of enzymatic activity precedes the observable cleavage of HF to make recognizable HFa.²⁸ The cleavage step occurs subsequent to kallikrein formation, and the latter enzyme acts upon HF in a feedback reaction. A further development of this hypothesis was proposed by Heimark et al²⁹ and by Kurachi et al³⁰ who observed rapid activation of prekallikrein and of factor XI by bovine HF and suggested that the initial rate of kallikrein formation was similar whether zymogen HF or HFa was used. These authors postulated that surface-bound HF was capable of a substrate-induced conformational change that created an efficient prekallikrein activator. This activity would not be detected by LMW peptide substrates that lack the macromolecular structures necessary to induce the conformational change.

Definitive proof of the creation of active sites prior to polypeptide chain cleavage is difficult to obtain. The reciprocal activations of HF and prekallikrein create a system with positive feedback that is therefore subject to rapid acceleration. Tankersley and Finlayson²⁵ estimated the kinetic constants for the component reactions and calculated that, at plasma concentrations of HF and prekallikrein, the introduction of a surface into the mixture where one molecule of active enzyme is present per milliliter ($5 \times 10^{-13}\%$ total protein) will result in 50% activation of either protein in 13 seconds. Such a low level of contamination of native HF with HFa has not been achieved; a level of 0.02% is the lowest thus far reported.³¹ Therefore, if native HF had no activity, rapid

activation of prekallikrein would be observed due to the HFa that is necessarily present.

Because human HF is able to autoactivate, low levels of HFa can accumulate in preparations of HF, even in the absence of a surface. Addition of irreversible inhibitors of active enzyme (eg, diisopropyl fluorophosphate, DFP) does not deplete active enzyme in a preparation much below 0.1%. We used a rapidly acting chloromethyl ketone inhibitor to try to destroy all traces of HFa in preparations of HF and thereby reduced enzymatic activity to less than 1/4,200 of that of HFa.³¹ Even after such treatment, HF was able to autoactivate and activate prekallikrein, albeit with very low initial rates. These experiments led to a proposal that the enzymatic activity of the zymogen (if any) is too low to measure and that the mechanism of contact activation of human plasma involved extremely low levels of active enzyme that are continuously present. That this might be possible *in vivo* is suggested by experiments in which prolonged incubation of purified HF with C1 esterase inhibitor (C1 INH) failed to prevent the conversion of HF to HFa or HF fragment (Hff).³² Therefore, the contact of plasma with a surface may induce "activation" by accelerating many 1,000-fold a normally occurring slow turnover of HF and prekallikrein. The effect of trace amounts of activity already present is amplified by the surface. The possibility that native HF does have trace activity analogous to the activity present in trypsinogen³³ is not excluded, although it is subject to the constraints of the aforementioned experiments. It should also be considered that the functioning of the contact system *in vivo* might require formation of trace quantities of HFa by other plasma enzymes, perhaps derived from cells.

There appear to be two components to the mechanism by which surface accelerates the reactions of contact activation. Griffin³⁴ demonstrated that the activation of HF by a series of different proteases was accelerated in the presence of a surface. He proposed that HF underwent a conformational change upon binding that increased its susceptibility to the cleavage leading to HFa formation. This observation suggests that surface-bound HF is a better substrate regardless of whether HFa (autoactivation) or kallikrein effects its cleavage and that this augmentation is one component of the catalytic action of the surface.

It is not known whether the cleavage and activation of surface-bound HF is accomplished by an enzyme molecule bound to a neighboring surface site or by one that remains free in solution. In the latter case, the catalytic effect of the surface would be due in large part to the creation of a local milieu in which the reversible binding of the protein components results in their increased concentration. Alternatively, the surface might physically locate the enzyme and the substrate in neighboring binding sites just as an enzyme does for its substrates. Another way to phrase this question is to ask whether both components of the Michaelis complex interact with the surface or only the substrate. Different mechanisms may be important for activation by HFa or kallikrein and with different surfaces.

Once a small amount of HFa has been produced, it converts prekallikrein to kallikrein,¹² the major plasma enzyme that activates HF.¹⁴ There is, therefore, reciprocal

activation in which these two proteins digest each other.³⁵ Because the rate of HF activation by kallikrein is much more rapid than HF activation by HFa,^{23,25} this pathway should be kinetically dominant. This conclusion is supported by studies of normal and prekallikrein-deficient plasma (Fig 1).

Recent evidence indicates that kallikrein also has to bind to the surface for maximum efficiency of HF activation. Thus, kallikrein cleaves surface-bound HF 3,000 times faster than fluid-phase HF, but cleavage by an equimolar quantity of the isolated (reduced and alkylated) light chain of kallikrein, which lacks the surface binding site,³⁶ is accelerated only 30-fold.³⁷ The activation of prekallikrein by HFa is also facilitated by surface, but to a lesser degree; in one study, dextran sulfate gave a 60-fold increase in cleavage by HFa and had no effect on the rate of cleavage by HFf.²⁵

It is pertinent at this point to consider the variety of compounds that have been used as surfaces. Much of the original work delineating the pathways of contact activation was done with kaolin. In terms of contact activation, however, kaolin is an ill-defined substance that is capable of binding many plasma proteins. More recently, dextran sulfate and sulfatide have been used as surfaces in studies of contact activation.^{24-26,30,31,38,39} These materials have the advantage of being much more homogeneous and, in the case of dextran sulfate, truly soluble. Using these activators, it has been possible to determine the rates of appearance of enzymatic activity and thereby to derive kinetic constants for the component reactions.^{24,25,37}

The idea of a reciprocal reaction between the zymogen and activated forms of HF and prekallikrein was complicated by the realization that there was yet another component required for optimal contact activation.^{40,41} Persons were studied whose plasma was not deficient in HF or prekallikrein, but their plasma gave very long kaolin-induced partial thromboplastin times (PTT) and generated no bradykinin. This led to the identification of HMW kininogen as a nonenzymatic cofactor that augments reciprocal activation.⁵⁻⁷ Initial experiments conducted with kaolin demonstrated that the presence of HMW kininogen increases the rate of conversion of prekallikrein to kallikrein by HFa and also increases the rate of activation of HF by kallikrein.^{42,43} HMW kininogen also increases the rate of factor XI activation by HFa.

One critical observation regarding the mechanism by which HMW kininogen functions as a cofactor was the demonstration that in plasma both prekallikrein⁸ and factor XI⁹ circulate bound to HMW kininogen and that these complexes bind to initiating surfaces via the HMW kininogen moiety.^{44,45} In plasma, HMW kininogen augments the attachment of prekallikrein and factor XI to surfaces such as kaolin^{44,45}; when the HMW kininogen is cleaved, this effect is augmented.^{46,47} Thus, HMW kininogen may be an activatable cofactor,⁴⁶ although the magnitude of this effect is uncertain. Adhesive glycoproteins such as fibrinogen may compete with HMW kininogen for binding to initiating surfaces. Studies have shown the presence of antigenic fibrinogen on clot-promoting surfaces that becomes undetectable after ten minutes of contact when HMW kininogen is present, an effect not observed in HMW kininogen-

deficient plasma.^{48,49} It will be of interest to determine whether cleaved and uncleaved HMW kininogen behave differently in such assays.

In buffer systems,⁴⁵ prekallikrein and factor XI bind to kaolin equally well in the presence or absence of HMW kininogen, but activation of each by HFa is dependent upon the presence of HMW kininogen. Aside from the quantitative binding effects seen in plasma, therefore, HMW kininogen must place the substrates of HFa in a conformation that facilitates their cleavage (Fig 2). More difficult to explain is the ability of HMW kininogen to augment the cleavage and activation of HF by kallikrein since it has no demonstrable effect upon the enzymatic activity of kallikrein (using synthetic substrates) and no known interaction with HF. The dissociation constant for the prekallikrein (kallikrein)-HMW kininogen complex is 15 nmol/L,^{50,51} and at plasma concentrations, about 10% to 20% of prekallikrein circulates free.^{8,50-53} Thus, kallikrein that is formed by activation of surface-bound prekallikrein-kininogen complexes can dissociate from the surface. It is then able to digest adjacent HF molecules on the same or other particles.^{45,54} In this fashion contact activation can be disseminated along the surface (Fig 2). Thus, the effective concentration ratio of kallikrein/HF is increased in the presence of HMW kininogen, which may account for the augmentation.

In contrast to these observations are studies of the human contact activation system that have been done with purified HF and prekallikrein by using dextran sulfate or sulfatide as activators. Under these conditions, both HF and prekallikrein are rapidly activated in the absence of HMW kininogen.^{24,25,31,37,55} The cofactor produces a small acceleration in the activation of HF by kallikrein,⁵⁵ whereas its effect on the reverse reaction has not been reported. This contrasts with activation in the presence of kaolin in which activation of prekallikrein by HFa is strongly dependent on HMW kininogen.⁴⁵ When dextran sulfate is added to plasma, however, almost no kallikrein is produced if HMW kininogen is absent.⁵⁶ Thus, the effects of a surface vis-à-vis the role of HMW kininogen differ somewhat depending upon the nature of the surface used and whether the reaction is studied in plasma or with purified components.

One way in which a plasma milieu might be different relates to observations that HMW kininogen may protect

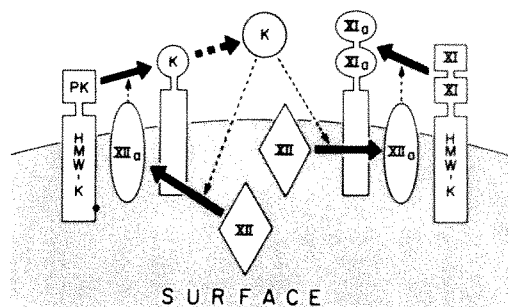


Fig 2. Hypothetical depiction of surface binding of HF (factor XII) and complexes of HMW kininogen (HMW-K) with prekallikrein or factor XI. Kallikrein dissociation to facilitate factor XII activation on the same or adjacent particles is shown by dashed lines.

kallikrein^{57,58} and factor XIa⁵⁹ from inhibition by C1 INH, α_2 macroglobulin, and α_1 antitrypsin and thereby augment the plasma half-life of each enzyme. The light chain of HMW kininogen, the portion of the protein that binds to prekallikrein and factor XI,⁶⁰ was reported to be as effective in preventing the inhibition as intact HMW kininogen. This result suggested that the phenomenon cannot be explained by substrate competition between HMW kininogen and C1 INH at the active site of kallikrein.⁵⁸ This is a controversial point, and Van der Graaf et al observed no effect of HMW kininogen upon inhibition of kallikrein by C1 INH.⁶¹ We found the second-order rate constant for the reaction of kallikrein with C1 INH to be unchanged in the presence of a saturating concentration of kinin-free HMW kininogen.⁶² Therefore, we are unsure whether HMW kininogen protection of enzyme inactivation contributes significantly to its augmentation of contact activation.

CLEAVAGE OF PROTEINS DURING CONTACT ACTIVATION

Figures 3 to 5 diagram the cleavages and fragments resulting from activation of each component of the system and indicate functional domains where they have been defined. HF (Fig 3) is initially cleaved within a disulfide bridge so that a single chain of 80,000 is converted to a two-chain enzyme in which a heavy chain of 50,000 is disulfide linked to a light chain of 28,000.¹⁷ No loss in size results, and the enzyme is called HFa (or α HFa). The binding site for the surface is on the heavy chain,⁶³ and the light chain contains the active enzymatic site. Further cleavage by kallikrein digests HFa at two sites (2 and 3) in sequence²³ to form HFf (or β HFa) in which a heavy chain of 28,000 is disulfide linked to a light chain of either 2,500 or 500.⁶⁴ A mixture of these two forms accounts for the doublet typically seen in alkaline disk gels¹² or unreduced sodium dodecyl sulfate (SDS) gels.¹⁷

Enzymes other than kallikrein are able to cleave and activate HF, including plasmin^{13,34,65} and factor XIa,¹⁴ but

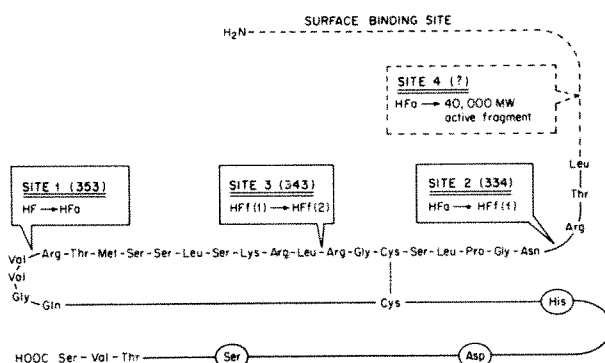


Fig 3. Sites of cleavage of HF during contact activation. Site 1, within a disulfide bridge, converts HF to HFa. Cleavage external to the disulfide bond at sites 2 and then 3 converts HFa to the two forms of HF. The light chain of HFf (residues 334 to 353 or 343 to 353) is derived from the C-terminal end of the heavy chain of HFa. Since the surface binding site is at the amino terminal end of HF or HFa, formation of HFf leads to dissociation from the surface. The active-site charge-relay complex (His, Asp, Ser) is at the C-terminal end.

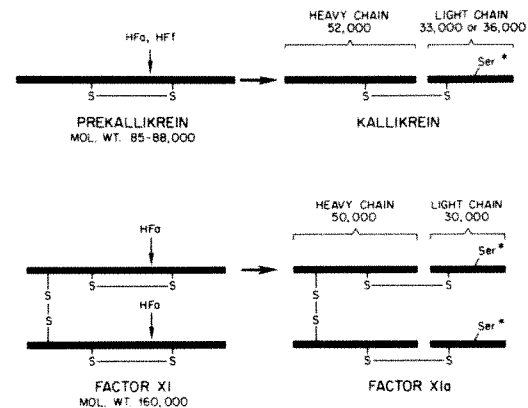


Fig 4. Diagrammatic representation of the activation of prekallikrein and factor XI by HFa. Cleavages occur within disulfide bridges in each case, and the active site is in the light chain(s). Because factor XI is dimeric, factor XIa has two identical active sites.

these reactions are less efficient. The cleavage of HF by HFa during autoactivation is of potentially greater significance than these reactions. Autoactivation produces, in addition to HFa and HFf, a third form in which the serine protease domain is linked to a 12,000-MW fragment of heavy chain to give a molecule of MW 40,000 before reduction^{13,23} (Fig 3), but kallikrein rapidly converts it to HFf.

The complete amino acid sequences of the heavy chain of HFa⁶⁶ and both chains of HFf⁶⁷ are now known. Fujikawa and McMullen⁶⁷ found nine amino acids in the light chain of HFf, presumably corresponding to the short segment remaining after cleavage at site 3. The heavy chain possesses a variety of structural domains with homology to other proteins. Two short domains resemble fibronectin, two growth factor regions are homologous with epidermal growth factor, and a kringle structure is seen, as has been reported in plasminogen, tissue plasminogen activator, urokinase, and prothrombin. The light chain of HFa (or heavy chain of HFf) containing the active site has a high degree of homology with tissue plasminogen activator and progressively less homology

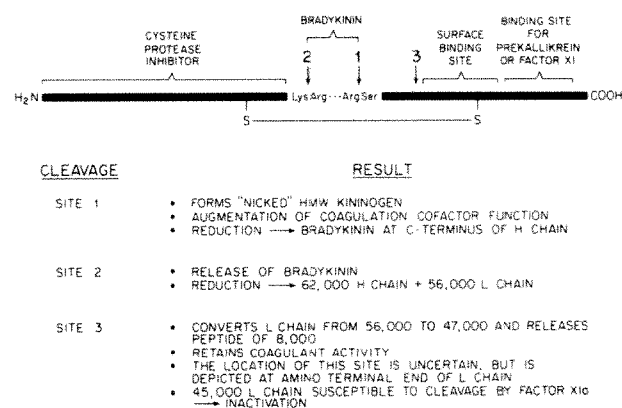


Fig 5. Depiction of the structure of human HMW kininogen and the consequences of cleavages at these sites. The amino terminal portion (heavy chain of cleaved kininogen) is a cysteine protease inhibitor; the light chain of cleaved HMW kininogen serves as the cofactor function in coagulation.

with urokinase, plasmin, trypsin, factor X, or factor IX. Strong homology with proteins involved in fibrinolysis is apparent. More recently, the complete amino acid sequence has been deduced by using factor XII cDNA isolated from a c-DNA library prepared from human liver mRNA.⁶⁸

Hff lacks the binding site for the surface and, once formed, is released into the fluid phase. It is not a coagulation factor since factor XI activation is strictly surface dependent⁶⁹; Hff possesses only 2% to 5% of the coagulant activity of HFa.^{12,13} Hff is an effective prekallikrein activator,^{12,13,69} however, and can lead to bradykinin formation until C1 INH binds and inactivates it.^{70,71}

Prekallikrein is a single chain γ globulin (isoelectric point [IEP] 8.9) with two forms at MW 85,000 and 88,000,⁷² both of which are present in all plasmas tested. As many as seven forms can be identified by isoelectric focusing.⁷³ Prekallikrein is converted to kallikrein (Fig 4) by cleavage within a disulfide bridge such that a heavy chain of 52,000 is disulfide linked to light chains of either 33,000 or 36,000 (one for each molecular form).^{72,74} The heavy chain interacts with the surface and binds to HMW kininogen,³⁶ whereas the light chain has the enzymatic active site.⁷² Purified light chain retains enzymatic activity on synthetic substrates³⁶ or in fluid-phase kinin generation but is ineffective as a surface-dependent coagulation factor. During lengthy incubations, the heavy chain of kallikrein is further cleaved so that a kallikrein of lower MW results (β -kallikrein), with little loss in kinin-forming ability; but activation of other proinflammatory actions (discussed subsequently) are altered.⁷⁵

Factor XI is unusual among coagulation factors because it consists of two identical disulfide-linked chains^{76,77} (Fig 4). Upon activation each chain is cleaved to yield disulfide-linked heavy and light chains; therefore, the active enzyme has four chains. The heavy chains bind to the surface.⁷⁸ Each light chain has an active site⁷⁹ and, when inactivated, can bind 1 mol of inhibitor.⁷⁷ Also, it has recently been shown that the molar ratio of binding of HMW kininogen to factor XI is 2:1.⁸⁰

HMW kininogen is cleaved by plasma kallikrein at two sites (Lys-Arg and Arg-Ser) to liberate the nonapeptide bradykinin. Both cleavages occur within a disulfide bridge⁸¹⁻⁸³ so that kinin-free HMW kininogen consists of a heavy chain of MW 62,000 disulfide linked to a light chain variously reported as having an MW of 56,000 to 62,000.^{46,50,84-87} It is unclear whether release of bradykinin results in chains that are equal in size (by SDS-polyacrylamide gel electrophoresis) and that another cleavage follows that forms the 56,000 band or whether the size differences reported in these studies are due to differences in technique. Further proteolysis of the light chain by kallikrein converts it to a light chain of 45 to 47,000.^{81,83,84} The heavy chain of HMW kininogen, which is identical to the heavy chain of LMW kininogen,^{81,88,89} has been shown to inhibit cysteine proteases⁹⁰⁻⁹² such as cathepsin L, papain, or platelet calpain 2.⁹³ In fact, cDNA analysis of α_2 thiol proteinase inhibitor demonstrates that it is identical to LMW kininogen.⁹¹ The light chain of HMW kininogen is the coagulant portion of the molecule^{60,81} and possesses a binding site for the surface⁹⁴ as well as a binding site for the heavy chain of prekallikrein^{8,51,52,60,95} or factor XI^{9,60,96} (Fig

5). These binding constants are estimated to be 3.4×10^7 mol/L⁻¹ and 4.2×10^8 mol/L⁻¹, respectively.⁶⁰

Human LMW kininogen is identical to HMW kininogen from the amino-terminus to 12 residues beyond the bradykinin moiety.⁹⁷ During transcription of kininogens the DNA corresponding to this segment can be spliced into two different DNA segments, which correspond to the kininogen light chains⁸⁹ (Fig 6). Those latter segments are completely different in terms of size, amino acid sequence, and function. LMW kininogen has no procoagulant activity and no known interaction with other proteins of the contact activation system, and the function of its light chain (if any) is unknown.

Bovine HMW kininogen has an additional kallikrein cleavage site located beyond the bradykinin moiety in a region that would correspond to the amino-terminal segment for the human HMW kininogen light chain. Cleavage at this site results in the loss of a fragment (termed fragment 1.2),⁹⁸⁻¹⁰⁰ which appears to contain the binding site for the surface.⁹⁴ Its removal thus destroys the coagulant cofactor activity.¹⁰⁰ The species difference may be attributable to the presence of a 22-residue sequence in the human but not the bovine protein and the presence of Lys rather than Arg at the site of the human protein analogous to the carboxy-terminus of bovine fragment 1.2.¹⁰¹ In humans, factor XIa performs a similar cleavage in the HMW kininogen light chain, which likewise inactivates it¹⁰² and may represent an intrinsic control mechanism.

The major kininogen of rodents is T kininogen from which a kinin (Ile-Ser-bradykinin) is cleaved by high concentrations of trypsin or cathepsin D¹⁰³ but not by kallikreins. The proteins corresponding to HMW and LMW kininogens are present but are minor components; all three kininogens are strongly homologous at the DNA and amino acid sequence level.¹⁰⁴

THE INTRINSIC FIBRINOLYTIC PATHWAY

An HF-dependent pathway leading to the conversion of plasminogen to plasmin was described in the 1960s and early 1970s.¹⁰⁵⁻¹⁰⁷ A defect in this pathway has been observed in plasma deficient in either HF, prekallikrein, or HMW kininogen.²⁻⁷ This is a relatively weak fibrinolytic pathway, and it is difficult to demonstrate in whole plasma because it does not lead to formation of large quantities of a potent plasminogen activator. Relatively small quantities of plasmin are generated, and the plasmin is then rapidly inacti-

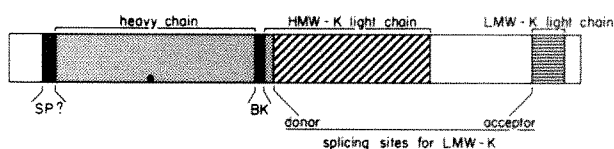


Fig 6. Diagram of the structure of the gene for the kininogens. A potential splicing site is located just beyond the bradykinin. If the DNA is read through this region, HMW kininogen m-RNA results. If the alternative splicing site and more distal exon is attached, LMW kininogen mRNA is formed.

vated by plasma inhibitors. Therefore, most of the aforementioned studies utilized diluted acidified plasma in which inhibition of both contact activation and plasmin are minimized and fibrinolytic activity can be measured. Although the identification of the plasminogen activator in this pathway has proved to be particularly difficult, certain aspects of the abnormal behavior of various deficient plasmas are predictable, given that HF is required. Prekallikrein and HMW kininogen are needed for optimal HF activation, and HMW kininogen is required for activation of both prekallikrein and factor XI; therefore, a deficiency of any of these proteins will be associated with abnormal intrinsic fibrinolysis.

By utilizing purified components, kallikrein was first shown to be a plasminogen activator.¹⁰⁸ A plasminogen proactivator was described subsequently that appeared to differ from prekallikrein¹⁰⁹; the activity was demonstrable in prekallikrein-deficient plasma.¹¹⁰ Others disagreed, reporting that plasminogen proactivator and prekallikrein were identical.^{111,112} Later studies, however, demonstrated that a plasminogen proactivator could be identified in the γ globulin fraction of prekallikrein-deficient plasma and was identified as factor XI.⁷² When compared, kallikrein and factor XIa were found to be equipotent as plasminogen activators.¹¹³ The concentration of prekallikrein, however, is much greater than factor XI (six- to tenfold); kallikrein can dissociate from surfaces into the fluid phase, whereas factor XIa does not, and prekallikrein can be activated in the fluid phase by HFF. Therefore, kallikrein appears to be more important than factor XIa as a plasminogen activator although one can demonstrate a fibrinolytic abnormality in factor XI-deficient plasma.¹¹⁴ The potency of both kallikrein and factor XIa on a molar basis is, however, many thousand-fold less than urokinase.^{113,115,116} Activated HF (HFa or HFF) also can convert plasminogen to plasmin directly,¹¹⁷ but it is only about 5% as potent as kallikrein.¹¹³ These are all weak reactions, and one can readily argue that plasminogen is not a significant substrate for any of these enzymes. It is interesting to note, however, that direct incubation of factors VIIa, IXa, Xa, or thrombin with plasminogen fails to generate plasmin at any concentration tested. Of the coagulation factors, then, only proteins of the contact activation system catalyze the conversion of plasminogen to plasmin. More recently, there have been reports that kallikrein can activate the trace of prourokinase present in plasma¹¹⁸ and urokinase might then be the major plasminogen activator.¹¹⁹ Inhibition by antiurokinase antibodies supports this notion,¹²⁰ whereas others disagree.^{120a} Nevertheless, the quantity of urokinase generated is very small. The effects of both C1 INH and α_2 antiplasmin may be abrogated by the addition of certain organic compounds (eg, flufenamic acid derivatives) to plasma.^{121,122} Contact activation of plasma treated in this way results in the formation of only about 35 ng/mL of plasmin¹²¹ (Fig 7).

INTERACTION WITH OTHER PLASMA PROTEASES AND WITH CELLS

HFa has been reported to activate factor VII^{123,124} and thereby initiate the extrinsic coagulation pathway. This

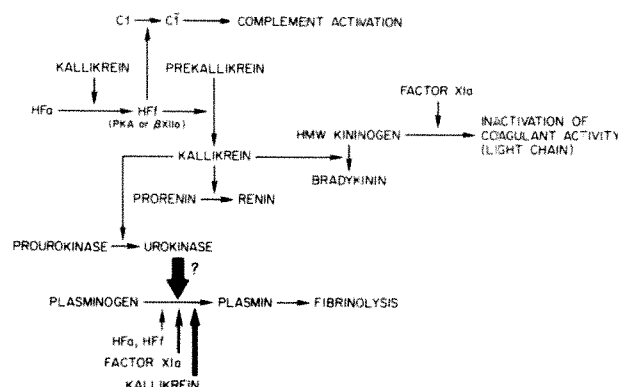


Fig 7. Interaction of proteins of contact activation with other plasma proteolytic cascades. HFF can initiate activation of the classic complement pathway, whereas kallikrein can convert prorenin to renin and participate in intrinsic fibrinolysis. Kallikrein, factor XIa, and HFa can all directly convert plasminogen to plasmin. Kallikrein can also convert single-chain urokinase (termed prourokinase; this is an active enzyme that is normally inhibited in plasma by a competitive inhibitor) to two-chain urokinase (which transiently escapes inhibition). Whether this urokinase is the major plasminogen activator or whether it is the direct effect of kallikrein is uncertain.

contributes minimally to the kaolin-activated PTT as usually performed, but it is responsible for the shortened clotting time seen when plasma is exposed to the cold.^{125,126} This is accentuated in women who use oral contraceptives containing estrogen, apparently due to an increased concentration of HF.¹²⁷⁻¹²⁹ It is theorized that this pathway might contribute to the increased incidence of thrombosis reported as a complication of oral contraceptive use.

HFF (but not HFa) has been shown to activate enzymatically the first component of complement¹³⁰ when it is incubated with purified C1 or added to plasma (Fig 7). C1 activation is due to cleavage of the C1r subcomponent¹³¹ by HFF. Little complement activation is seen, however, when kaolin is incubated with whole plasma. Significant complement activation may be seen only in conditions that result in substantial conversion of HFa to HFF. One such circumstance is C1 INH deficiency (ie, hereditary angioedema) in which HF activation may contribute to complement consumption.^{132,133} Kallikrein can also cleave C1 subcomponents, but the net result is destruction rather than activation. On the other hand, kallikrein can activate factor B of the alternative complement pathway and thereby substitute for factor D.¹³⁴

Kallikrein has been reported to interact with human leukocytes in a variety of ways. It is a chemotactic factor for neutrophils¹³⁵ and monocytes,¹³⁶ and it has been shown to cause neutrophil aggregation¹³⁷ and release of elastase.¹³⁸ In a rabbit model, kallikrein stimulation of chemotaxis appeared to require cleavage of C5 and release of C5a chemotactic factor.¹³⁹ It is therefore possible that C5 bound to the surface of neutrophils is being cleaved in the aforementioned reactions. However, antikallikrein serum was inhibitory whereas anti-C5 serum had no effect; the authors therefore concluded that the effect of kallikrein on human neutrophils is independent of complement. Further, a degraded form of kallikrein (β -kallikrein) in which the heavy

chain is partially digested is enzymatically active on kininogen to form kinin, but its reactivity with neutrophils is markedly attenuated.¹⁴⁰ HFa (but not HFF) has also been shown to stimulate neutrophils; a requirement for a binding site in the heavy chain appeared to be required for the reaction to proceed.¹⁴¹ There are no studies demonstrating either a cell surface receptor for these enzymes or required cleavage of surface components. In each instance, however, the active site of the enzyme is required so that the proenzyme or DFP-treated enzyme is inactive.^{135,137}

The components of the contact activation system also appear to interact with platelets. There are apparently separate platelet receptors for plasma factor XI and factor XIa.¹⁴² Platelets also have intrinsic factor XI activity that differs from plasma factor XI in MW and isoelectric point but that cross-reacts with it immunologically. This activity is present in patients who are deficient in plasma factor XI.^{143,144} Platelet-associated factor XI can be activated by both HF-dependent and -independent mechanisms.¹⁴⁵ The membrane of activated platelets also appears to provide a surface on which HF can be activated, a process dependent on prekallikrein and HMW kininogen,¹⁴⁵ so that bound factor XI is then activated. HMW kininogen demonstrates binding to platelets^{146,147} that is zinc dependent.^{147a} It has been shown to be present within the alpha granules of platelets,¹⁴⁷ becomes available during platelet activation,¹⁴⁸ and augments binding of factor XIa.¹⁴² Yet it is clear that platelets are not requisite for contact activation as they are for later steps in coagulation. Therefore, contact activation proceeds normally in platelet-poor plasma, and the addition of platelets provides little or no augmentation. In fact, there is evidence that platelets may actually inhibit prekallikrein and factor XII activation by secretion of β thromboglobulin^{148a,148b}; however, platelet activation in peripheral tissues due to injury and/or localized thrombosis may contribute to contact activation and local kinin formation. In some circumstances the platelets may also provide a factor XII bypass in contact activation.¹⁴⁹

CONTROL MECHANISMS

Regulation of the HF pathways occurs by both intrinsic and extrinsic mechanisms. Cleavage of HFa to HFF is one example of an intrinsic control in which coagulation via the surface-dependent activation of factor XI is limited whereas bradykinin generation via fluid-phase activation of prekallikrein continues. Likewise, the digestion of kinin-free HMW kininogen light chain by factor XIa limits its coagulant activity.¹⁰² Extrinsic controls are provided by plasma inhibitors of each enzyme (Table 1). The only major plasma inhibitor of HFa or HFF is C1 INH.^{70,71,150,151} There are two main kallikrein inhibitors in plasma: α_2 macroglobulin¹⁵² and C1 INH.^{153,154} When kallikrein is added to plasma, approximately half is bound to each inhibitor at equilibrium.^{155,156} When a surface such as kaolin is added to plasma, however, close to 70% or 80% of the kallikrein formed is bound to C1 INH¹⁵⁷; the mechanism responsible for this change in inhibition ratio is unknown. Factor XIa is inhibited primarily by α_1 antitrypsin^{59,158} and C1 INH,¹⁵⁰ whereas plasmin is inhibited by α_2 antiplasmin¹⁵⁹⁻¹⁶² and α_2 macroglobulin.¹⁶³⁻¹⁶⁵ Anti-

Table 1. Major Inhibitors of Active Proteases of the Contact Activation System

Enzyme	Inhibitor
HFa (HFF)	C1 INH
Factor XIa	α_1 Antitrypsin, C1 INH
Kallikrein	C1 INH, α_2 macroglobulin
Plasmin	α_2 Antiplasmin, α_2 macroglobulin

thrombin III (AT III) accounts for only a few percent of plasma inhibition of each of these factors,¹⁶⁶⁻¹⁷¹ but its contribution in the presence of heparin is uncertain. There is clearly less augmentation than is seen with thrombin. Some studies have revealed little augmentation of AT III inhibition of HFa,^{71,151,172} kallikrein,¹⁷³ or factor XIa¹⁷⁴ in the presence of heparin, whereas others find that heparin has a prominent effect upon the rate of AT III inhibition of each of them.^{168,171,175} These reactions need to be reassessed with purified components and the rate of inhibition of each enzyme carefully determined in heparinized *v* citrated plasma. For such studies the dose of heparin and definition of the activity of the fraction used will be critical.

Bradykinin is an exceedingly potent vasoactive peptide that can cause venular dilatation, increased vascular permeability, hypotension, constriction of uterine and gastrointestinal (GI) smooth muscle, constriction of coronary and pulmonary vasculature, bronchoconstriction, and activation of phospholipase A₂ to augment arachidonic acid mobilization. Its regulation is of prime importance, and plasma contains a variety of enzymes that serve this function. Recent studies of kinin degradation from our laboratory are summarized in Fig 8. First, the C-terminal arginine is removed by carboxypeptidase N,^{176,177} thereby leaving the residual octapeptide. The des-Arg⁹-bradykinin formed is inactive in the skin or GI tract, but it is equipotent to bradykinin in causing hypotension.¹⁷⁸ Separate receptors for these two moieties have been proposed and are called B₁ (des-Arg⁹-bradykinin) and B₂ (bradykinin).¹⁷⁹ The rate of Arg removal in serum, however, far exceeds the rate in plasma,¹⁸⁰ whereas the level of carboxypeptidase N is unaltered. It appears that a carboxypeptidase B-like enzyme is generated during coagulation¹⁸⁰ that is secreted, perhaps, by platelets or neutrophils. Des-Arg⁹-bradykinin is then degraded by angiotensin-converting enzyme to the pentapeptide Arg-Pro-Pro-Gly-Phe plus the tripeptide Ser-Pro-Phe.¹⁸¹ The C-terminal phenylalanine is then cleaved from each peptide by enzymes possessing

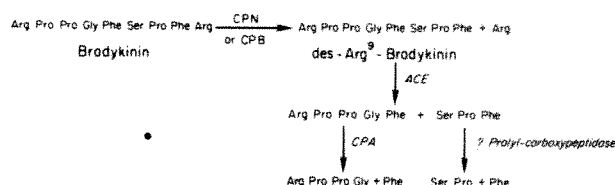


Fig 8. Pathway for degradation of bradykinin in human plasma. One or more carboxypeptidases remove the C-terminal arginine, and des-Arg⁹-bradykinin is cleaved by angiotensin-converting enzyme (ACE). The pentapeptide and tripeptide products are further degraded by enzymes (not yet characterized) with activities resembling carboxypeptidase A and/or a prolylcarboxypeptidase.

activities consistent with carboxypeptidase A and prolylcarboxypeptidase, respectively.¹⁸⁰ These enzymes have not yet been isolated or characterized. Not shown in Fig 8 is the further clearance of Arg-Pro-Pro-Gly and Ser-Pro so that the final degradation products of bradykinin digestion are Arg-Pro-Pro-Gly and free amino acids.

Because bradykinin is a peripheral vasodilator, it has been considered to act as a potential "counterbalance" to the vasopressant effects of angiotensin II. From the previous discussion, it is clear that these two peptides are also related in terms of metabolic control since angiotensin-converting enzyme cleaves the decapeptide angiotensin I to generate angiotensin II and also degrades and inactivates bradykinin or des-Arg⁹-bradykinin. It is important to point out that although angiotensin-converting enzyme readily degrades bradykinin in physiological buffers,¹⁸² bradykinin is first converted to des-Arg⁹-bradykinin in plasma or serum and the latter peptide is actually the substrate for the converting enzyme.

There are other interactions of the kallikrein/kinin system and the renin/angiotensin system in plasma and serum that have been described. For example, plasma kallikrein has been shown to convert prorenin to renin (Fig 7)^{183,184}; when plasma is acidified and then neutralized, prorenin is converted to renin, and the latter alkaline phase is an HF-dependent reaction. It appears that acidification negates the effects of plasma inhibitors and affects prorenin susceptibility to activation. The importance of this reaction under normal physiological conditions is uncertain.^{185,186,186a}

ASSESSMENT OF CONTACT ACTIVATION IN HUMAN DISEASE

The HF-dependent pathways described herein are not likely to contribute to thrombosis and hemostasis in a major way. Thus, although activation of HF leads to *in vitro* coagulation, in most thrombotic disorders there is activation of the extrinsic pathway occurring either alone or in addition to HF activation, and the effects of tissue factor predominate. Certainly, there is no thrombosis associated with hereditary angioedema, a disorder in which contact activation is prominent. Likewise, patients deficient in HF, prekallikrein, and HMW kininogen have no obvious bleeding diathesis, and factor XI-deficient patients have a bleeding disorder that is highly variable and often mild. Observations such as these have led to considerations of physiological mechanisms of factor XI activation that bypass the other contact activation components, but these remain unsettled. Most evidence suggests that the importance of the cascade relates to the pathogenesis of inflammatory reactions, the local control of blood flow (in which bradykinin functions as a hormone), and perhaps control of BP. To assess the HF-dependent pathways in human disease, we ideally would like to measure each active enzyme, demonstrate cleaved HMW kininogen, and assay bradykinin. These determinations are limited because the enzymes and the bradykinin are rapidly inactivated, and often one has to determine the level of residual proenzyme. One can quantitate HF, prekallikrein, or HMW kininogen in plasma by coagulant assay,¹⁸⁷ immunologic determination of antigen levels,^{53,188,189} or by *in vitro* activa-

tion and evolution of enzymatic activity.^{38,190-192} Shifts in electrophoretic mobility are indicative of complex formation between enzymes and their inhibitors and provide qualitative evidence that activation has occurred. Thus far monoclonal antibodies that react solely with the active site of enzymes have not been developed, although De Agostini et al¹⁹³ have described a monoclonal antibody that reacts with a neoantigen in the kallikrein-C1 INH complex.

The previously described assays are useful if sufficient activation has occurred to cause significant depletion of substrate. But if only a small amount of each factor is converted, it may be missed. Assays have recently been developed for enzyme-inhibitor complexes based on the assay for plasmin- α_2 antiplasmin complexes by Harpel.¹⁹⁴ These include double-antibody enzyme-linked immunosorbent assay methods for HFa-C1 INH,¹⁹⁵ kallikrein-C1 INH,¹⁹⁶ and kallikrein- α_2 macroglobulin complexes,¹⁹⁷ which can detect as little as 1% activation of any of the components. Bradykinin¹⁹⁷ and des-Arg⁹-bradykinin¹⁹⁸ can be determined by radioimmunoassay.

These latter assays have just begun to be applied to disease states; most of the literature describes results obtained with earlier methods. Hereditary angioedema receives considerable attention because C1 INH is absent and there is simultaneous activation of the HF-dependent pathways and the classic complement pathways. Patients present with episodic swelling occurring virtually anywhere in the body, attacks of severe abdominal pain due to edema of the bowel wall, and laryngeal edema. Although a kinin derived from a cleavage product of the second complement component was thought to be responsible for the edema,¹⁹⁹ other studies suggest that bradykinin is the critical peptide.^{133,200} On the other hand, a kininlike vasoactive substance may yet be shown to be a complement derivative, as shown by recent reports.^{200a} During attacks of angioedema there is depletion of prekallikrein and HMW kininogen²⁰¹ and formation of bradykinin,²⁰² and blisters induced in the skin of patients are found to have elevated plasma kallikrein levels.²⁰³ The complement activation appears due to autoactivation of C1 (specifically C1r) in the absence of C1 INH,^{204,205} but this may be augmented by HFF,^{130,131} particularly during acute attacks of swelling.

Aside from hereditary angioedema, there is a relatively large amount of literature demonstrating activation of the HF-dependent pathways in plasma or serum in a wide variety of inflammatory disorders; in some disorders, the activation is coincident with the patients' symptoms. There are no data, however, that prove that the consequences of such activation lead to disease manifestations or the converse, that inhibition of the pathway will ameliorate symptoms. These should be viewed as promising associations and are important areas for future investigation. These will only be briefly described.

Contact activation may occur during allergic diseases, for example, nasal washings of patients with allergic rhinitis who have been challenged with antigen contain lysyl bradykinin, bradykinin, kininogen, and kallikreins derived from mast cells and plasma.²⁰⁶⁻²⁰⁹ The latter may represent contact activation on a surface altered by the initial inflammation.

Other possibilities for activation might include negatively charged macromolecules in mucosal secretions or mast cell heparin.²¹⁰ Although we routinely utilize dextran sulfates as model compounds upon which HF activation can occur, other sulfated mucopolysaccharides may serve similar functions in vivo.^{211,212} The synovial fluid of patients with rheumatoid arthritis has been shown to contain plasma kallikrein, which has been shown to activate procollagenase to collagenase.²¹³ Uric acid and pyrophosphate crystals can act as surfaces for contact activation^{214,215} and may contribute to the inflammation of gout and pseudogout. A role for the contact activation system in inflammatory arthritis is therefore possible and may involve other functions of the enzymes formed in addition to any effects of kinins.

Activation of HF is also induced by the lipid A component of endotoxin,²¹⁶⁻²¹⁸ and contact activation proteins are depleted in endotoxic shock.²¹⁹⁻²²² The pooling of fluid into body cavities, the intravascular volume depletion, and the hypotension seen may be caused by release of bradykinin. Likewise, other bacterial infections such as typhoid fever have been shown to be associated with prekallikrein depletion, formation of kallikrein-C1 INH complexes, and C1 INH depletion as evidence of contact activation.²²³ In patients hospitalized because of trauma, the onset of sepsis has been shown to be associated with kininogen depletion, and determination of serial kininogen levels had prognostic value.²²⁰ An unfortunate circumstance has dramatized the kinin-forming capacity of HFf; trauma patients given plasma protein fractions (as plasma expanders) that were contaminated with HFf showed profound hypotension.^{224,225}

In patients with disseminated intravascular coagulation (DIC) due to endothelial injury and/or endotoxemia (including gram-negative sepsis, gram-positive sepsis, or viremia), decreased levels of plasma factor XII and prekallikrein, and kallikrein inhibitory activity are seen²²⁶; these changes were not observed in DIC associated with leukemia, carcinoma, or abortion.²²⁷ The data suggest activation of the HF-dependent pathways. A similar pattern of protein depletion has been observed in some patients with polycythemia vera,²²⁸ type II hyperlipidemia (familial hypercholesterolemia),²²⁹ and Rocky Mountain spotted fever.²³⁰ In some patients with nephrotic syndrome, a similar diminution of contact activa-

tion factors has been reported²³¹ that could not be explained simply by a loss of proteins in the urine.²³² Cirrhosis, as might be anticipated, is associated with diminished levels of plasma prekallikrein, HMW kininogen, and to a lesser degree, HF, which seems to be due to a diminished rate of protein synthesis.²³³

CONCLUDING COMMENTS

Considerable progress has been made in understanding the consequences of activation of the HF-dependent pathway of plasma. The various proteins and the functions of each have been characterized; yet we have little insight regarding the functions in normal homeostasis and are just beginning to appreciate the pathogenic roles in various inflammatory conditions and shock states.

The fact that patients deficient in HF, prekallikrein, or HMW kininogen do not bleed can be considered as evidence against the importance of contact activation in hemostasis. On the other hand, coagulation is one of the major consequences of HF activation and is the initiating stimulus every time a PTT is performed. Indeed, our concepts regarding bleeding per se are not well developed (particularly for spontaneous bleeding), and it is even conceivable that the absence of one or more contact activation factors (or plasma bradykinin) is protective against bleeding. Likewise, although factor XI deficiency is considered mild compared with factor VIII or IX deficiency, understanding why some patients do or do not bleed with very low levels of factor XI may provide an important piece of information. Areas in which bradykinin may be important in terms of normal physiology include control of the microcirculation in peripheral organs, particularly glandular tissue, and control of BP as a counterbalance to the renin-angiotensin system.

It appears likely that contact activation occurs in a variety of inflammatory conditions and may contribute to disease manifestations such as increased vascular permeability, angioedema, recruitment of polymorphonuclear leukocytes, and changes in BP. With new, more sensitive and specific assays for the components of contact activation, bradykinin, and their various inactivated and/or degradation products should come new insights regarding their roles in disease pathogenesis and new approaches to their inhibition.

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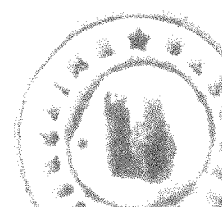
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A Human Monoclonal Autoantibody Recognizes a Neoantigen on Glycoprotein IIIA Expressed on Stored and Activated Platelets

By Diane J. Nugent, Thomas J. Kunicki, Cicely Berglund, and Irwin D. Bernstein

We prepared a heterohybrid cell line that secretes a human IgM monoclonal autoantibody that recognizes an antigen found on thrombin-activated or stored platelets. The surface expression of the epitope recognized by this autoantibody, 5E5, increases with time as platelets age in vitro, suggesting that it may represent a senescence or activation-specific antigen. 5E5 binds to the purified platelet membrane glycoprotein (GP) IIb-IIIa complex in an enzyme-linked immunosorbent assay (ELISA). In an immunoblot technique, 5E5 binds to a protein with an apparent mol wt of 95,000, which is identical to that of GPIIIa under

nonreduced conditions. In crossed immunoelectrophoresis (CIE), the predominant antigen recognized by 5E5 is contained in the GPIIb-IIIa precipitin arc. An additional precipitin arc recognized by 5E5 is often observed only on gels derived from lysates of platelets stored under blood bank conditions for >3 days. These findings illustrate the usefulness of human monoclonal antibodies for the identification of membrane neoantigens expressed as a result of platelet activation or revealed as platelets age in vitro.

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CELLS OF hematopoietic origin have a well-defined lifespan in blood circulation that is unique for each lineage. Platelets, for example, circulate for 7 to 10 days before they are removed by the reticuloendothelial system, whereas the life span of RBCs in circulation is much longer at 120 days.¹⁻³ Selective removal of senescent hematopoietic cells suggests the existence of epitopes that are expressed on the cell surface only after aging. These antigens could be generated by various processes, such as proteolytic degradation, desialation, or activation. The presence in normal sera of naturally occurring autoantibodies that react with aged erythrocytes or platelets and the increased phagocytosis of aged cells by autologous monocytes following incubation in such sera have led some investigators to propose that immune-mediated clearance is an important mechanism in the removal of senescent cells.⁴⁻⁸

In studies of platelet surface antigens, murine monoclonal antibodies have proved invaluable for identification, characterization, and purification of specific membrane proteins or glycoproteins.^{9,10} These antibodies recognize interspecies differences, however, and they have not yet proved helpful for defining subtle membrane protein changes associated with aging that may be more likely to be recognized by human autoantibody. To characterize the specific membrane changes that lead to the expression of senescence antigens recognized by antiplatelet immunoglobulin in humans, methods to isolate large quantities of monospecific human autoantibody are necessary. Shoenfeld and co-workers¹¹

were among the first to isolate cell lines secreting human monoclonal antibody used to characterize the distribution of an autoantigen associated with systemic lupus erythematosus. Using a similar approach, we transformed B lymphocytes from a patient with immune-mediated thrombocytopenic purpura (ITP) using Epstein-Barr virus (EBV), and isolated B cell lines that secrete human monoclonal autoantibodies that bind to platelet antigens. We now report the characterization of one such human monoclonal autoantibody, 5E5, that recognizes a surface antigen expressed on platelets aged in vitro.

MATERIALS AND METHODS

Patient data. The bone marrow cells used in this study were obtained from a 1-year-old white female, after informed consent was obtained, as part of a routine diagnostic marrow aspiration to confirm the clinical impression of immune-mediated thrombocytopenic purpura (ITP). The patient had never been transfused with any blood product; EBV and cytomegalovirus (CMV) serologic studies were negative at both the initial visit and 6 weeks thereafter. The bone marrow showed increased numbers of megakaryocytes but was otherwise normal. Laboratory studies confirmed the presence of circulating platelet-reactive IgM, but no reactive IgG or IgA. The platelet count was consistently <5,000/mm³, thus preventing adequate evaluation of platelet-associated immunoglobulin.

EBV transformation. Bone marrow mononuclear cells were depleted of T cells using 2-aminoethylisothiuronium bromide (AET)-treated sheep RBCs (Sigma, St Louis). The remaining cells were infected with EBV by coinubation with the EBV-secreting cell line 1A2 (provided by M. Lostrom, Seattle) as previously described.¹² After 2 weeks, culture supernatants from wells containing transformed lymphocytes were screened for the presence of antiplatelet antibody using a solid-phase ¹²⁵I-protein A binding assay.¹³ Cells from positive wells were expanded and hybridized with a 6-thioguanine-resistant, mouse-human heterohybrid line, F6.¹⁴ The fused cells were placed in 96-well plates at 10⁵ cells/well and cultured in selective medium containing 15% fetal calf serum (FCS) in RPMI (GIBCO, Grand Island, NY), 0.01 mmol/L of hypoxanthine, 10⁻⁵ mol/L of aminopterin, 20 mmol/L of thymidine (HAT) to eliminate nonfused F6 cells, and 10⁻⁶ mol/L of ouabain to eliminate nonfused transformed lymphocytes. After 2 additional weeks, wells containing hybrid cells were screened for the presence of antiplatelet antibody, and cells from positive wells were cloned by the limiting dilution technique.

Antibodies. Two human monoclonal antibodies, 55 and 3H8, are used as isotype identical control antibodies in this study. Antibody 55 was isolated from peripheral blood lymphocytes from a

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patient with malignant melanoma fused directly with F6 and is reactive with fibroblasts and melanoma cells but not platelets. Antibody 3H8 was used as a platelet-reactive positive control and was isolated from EBV-transformed splenic B lymphocytes from a 10-year-old patient with ITP. Both 55 and 3H8, like 5E5, are IgM molecules and bind staphylococcal protein A directly.

Indirect ^{125} I-Staphylococcal protein A platelet binding assay. To demonstrate binding of 5E5 to platelets aged in vitro, platelets were serially drawn from five individual donors at 96 hours, 48 hours, and 4 hours prior to incubation with the antibody preparation. In the interim between phlebotomy and antigen assays, platelet-rich plasma (PRP) fractions anticoagulated with acid citrate dextrose (ACD) (NIH Formula A) were stored sterilely in Fenwal PL-146 bags on an orbital platform rocker (70 cycles/min) at 22°C. In preparation for the platelet antibody binding assay,¹³ fresh or stored platelets were washed three times in Ringer's citrate dextrose (RCD), pH 6.8, and then pelleted by centrifugation. Two hundred microliters of the washed platelet suspension (2×10^5 platelets/ μ L) were incubated in RCD with 10 μ g of purified 5E5 or pooled human IgM (Cappel, Cochranville, PA) at 22°C for 60 minutes. Platelets were then washed twice in RCD, incubated with ^{125} I-staphylococcal protein A (10^5 cpm/sample) at 22°C for 30 minutes, washed twice again in RCD, and then counted in a γ counter (Packard Instruments, Downers Grove, IL).

Immunoblot of platelet proteins. Reactivity of human antiplatelet antibodies with platelet proteins was determined using the immunoblot technique described by Moroi and Burnette.^{15,16} In brief, 200 μ g of sodium dodecyl sulfate (SDS)-solubilized, ^{125} I-labeled protein, derived from 72-hour-old platelets outdated at the Puget Sound Blood Center, were electrophoresed under nonreducing conditions in an 8% polyacrylamide resolving gel using the discontinuous buffer system of Laemmli.¹⁷ The separated platelet proteins were then transferred to nitrocellulose paper using a Transblot apparatus (BioRad, Richmond, CA). After blocking with 1% gelatin (Sigma, St Louis) in 150 mmol/L of NaCl, individual strips of the nitrocellulose paper were incubated with each antiplatelet antibody or human serum for 60 minutes at ambient temperature, washed twice with 0.5% gelatin, 150 mmol/L of NaCl, 0.1% Triton X-100 (washing buffer), and then incubated with horseradish-peroxidase-linked goat anti-human IgM and IgG (Tago, Burlingame, CA). Immunoreactive protein recognized by enzyme-linked goat anti-human Ig was detected by developing the strips with the chromogen 4-chloro-1-naphthol dissolved in methanol, stock solution of 3 mg/mL. One volume of the stock solution was mixed with 5 vol 50 mmol/L of Tris-HCl, 200 mmol/L of NaCl, pH 7.4, and 0.015% H_2O_2 . Autoradiography of the strips was also performed to assure proper alignment of the platelet proteins.

Indirect immunofluorescence analysis of antibody binding to platelets. As described elsewhere by Andrews and colleagues,¹⁸ peripheral blood erythrocytes, mononuclear cells, granulocytes, and platelets were isolated and suspended in RCD to screen for surface binding of human monoclonal autoantibodies. Cells were incubated for 60 minutes at 22°C with either 5E5, 55, or pooled human IgM (10 μ g antibody/mL), washed twice in RCD, and incubated with fluoresceinated goat F(ab')₂-antihuman IgM (1:40 dilution) for 30 minutes at room temperature. The cells were washed twice with RCD and prepared for analysis by flow microfluorimetry (FACS 440 fluorescence-activated cell sorter, Becton Dickinson, Sunnyvale, CA).

Crossed immunoelectrophoresis. CIE was performed as previously described by Kunicki and co-workers.¹⁹ One hundred micrograms of lysate protein derived from fresh platelets or platelets stored under blood bank conditions for 72 hours at 22°C were electrophoresed at 10 V/cm for 60 minutes at 15°C in first-dimension gels consisting of 1% agarose and 0.5% (vol/vol) Triton

X-100 in 0.034 mol/L of Tris and 0.1 mol/L of glycine, pH 8.7 (Tris-Glycine). Electrophoresis in the second dimension was performed at 2 V/cm for 18 hours at 15°C. The second dimension consisted of a biphasic gel system with an intermediate gel containing 40 μ g/cm² ^{125} I-labeled human monoclonal antiplatelet antibody (either 5E5 or 3H8) and an upper gel containing the IgG fraction of polyclonal rabbit antiplatelet antisera (700 μ g/cm²). Gels were then washed, dried, and prepared for autoradiography as described.¹⁹

Platelet activation. Gel-filtered platelets were isolated from a PRP fraction of fresh whole blood collected in one-tenth volume ACD (formula A). The columns were prepared using sepharose 2B-CL (Pharmacia, Piscataway, NJ) equilibrated with modified Tyrode's buffer: 138 mmol/L of NaCl, 29 mmol/L of KCl, 0.42 mmol/L of $NaHPO_4$, 12 mmol/L of $NaHCO_3$, 0.5% bovine serum albumin (BSA), and 0.1% glucose, pH 7.4. Identical aliquots of these platelets were then exposed to either thrombin (final concentration of 0.5 U/mL) or Tyrode's buffer for 5 minutes under nonstirring conditions at ambient temperature. EDTA was not included in any of the buffers used in this experiment. Hirudin (final concentration of 4 U/mL) was added to both fractions followed by the addition of an equal volume of 2% paraformaldehyde (final concentration of 1% paraformaldehyde). After 30 minutes on ice, the fixed platelets were washed twice in RCD, and indirect immunofluorescence assays were performed as described above.

Platelet glycoprotein IIb-IIIa- or Ib-specific ELISA screening assay. Using a modification of the procedure described by Furihata and co-workers,²⁰ 1–5 μ g/mL purified platelet glycoprotein IIb-IIIa or Ib in carbonate buffer, pH 9.5, was applied to the surface of flat-bottom microtiter wells and incubated overnight at 4°C. Following two washes with PBS, 100 μ L of blocking buffer containing 1% gelatin, 135 mmol/L of NaCl, pH 7.4, was added to each well and incubated at room temperature for 30 minutes. The plates were washed twice with PBS, 0.05% Tween, pH 7.4 (PBS-Tween). Test monoclonal antibodies, pooled human IgM, or human plasmas at various dilutions were then applied in duplicate on each plate, and plates were incubated at room temperature for 60 minutes. Following two washes with PBS-Tween, a horseradish peroxidase-linked goat F(ab')₂ anti-human IgM and IgG was added at a dilution of 1:2000 in PBS-Tween and incubated for 30 minutes at room temperature. Bound antibody was detected using a 0.1 mol/L of sodium citrate buffer, pH 4.4, containing 0.015% H_2O_2 and 5 mg/10 mL of 2,2-Azinobi's (3-Ethylbenz-thiazoline sulfonic acid) (ABTS; Sigma) as the chromogenic agent.

RESULTS

Bone marrow lymphocytes obtained from a patient with ITP were cultured with EBV-secreting 1A2 cells. After 2 weeks in culture, 384 wells (76.8%) contained EBV-transformed lymphocytes. Using an indirect ^{125} I-staphylococcus Protein A binding assay, supernatant fluids in 16 (4%) of the 384 wells were shown to contain antiplatelet antibody. One of the antiplatelet antibodies, 5E5, which was of the IgM isotype, was selected for further characterization, hybridized with F6, and cloned using limiting dilution techniques. This line grows stably in culture and secretes 20 to 30 μ g/mL of antibody when cultured overnight at a cell density of 5.0×10^5 /mL.

Using an indirect immunofluorescence assay and flow microfluorimetry, the surface reactivity of 5E5 with whole blood elements was compared with a non-platelet-binding negative control human antibody 55 and to pooled human IgM. 5E5 did not bind to normal peripheral blood mononu-

clear cells, granulocytes, or erythrocytes, but did bind strongly to the surface of ~70% of the platelets (Fig 1). This pattern of reactivity was consistently observed whether single donor or pooled platelet preparations were used.

As outdated platelets (>72 hours old, Puget Sound Blood Center, WA) were initially used for screening in both the radioimmunoassay and microfluorimetry experiments, studies were performed to test the possibility that there may be a progressive increase in the expression of this antigen during in vitro aging and/or platelet activation. Using the ^{125}I staphylococcal Protein A binding assay, surface reactivity of 5E5 with platelets derived from five individuals was measured during the course of storage of these cells in vitro. The results of this experiment are illustrated in Fig 2 and represent mean values $\pm$ SD for five separate experiments. We observed negligible binding of 5E5 to fresh resting platelets as compared with an isotype identical negative control, 55, or pooled human IgM. With in vitro storage, however, there was a four- to five-fold increase in expression of the platelet antigen recognized by 5E5 at 48 and 96 hours, whereas there was still minimal binding of the two negative control antibodies to these aged platelets. Furthermore, by indirect immunofluorescence assays, fresh thrombin-stimulated platelets expressed the epitope recognized by 5E5 (Fig 3); again, ~60% to 70% of platelets were positive for this antigen in the activated platelet population.

Reactivity of 5E5 for GPIIb-IIIa was demonstrated in an ELISA system using plates coated with purified platelet GPIIb-IIIa or GPIb, as shown in Fig 4. In this study, 5E5 reacted specifically with GPIIb-IIIa but not with GPIb, whereas pooled human IgM and six other platelet reactive human monoclonal autoantibodies at similar IgM concentrations did not show binding to either of these adsorbed glycoproteins. Using human plasmas containing alloantibodies known to react with either GPIIb-IIIa (anti-PI^{A1}) or GPIb (anti-PI^{E1}), we were able to confirm the antigen specificity of this assay.

An immunoblot technique was used to identify any platelet proteins recognized by 5E5 (Fig 5). Protein derived from

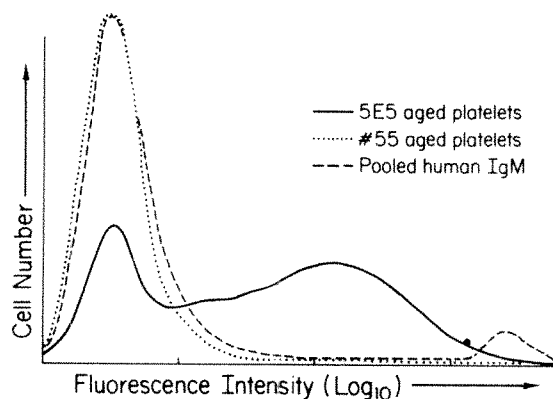


Fig 1. Indirect immunofluorescence analysis of antibody binding to stored platelets using flow microfluorimetry. The reactivity of 5E5 was compared with a nonplatelet binding negative control autoantibody, 55, and pooled human IgM. The autoantibody 5E5 binds to an epitope present on only 70% of the platelet population.

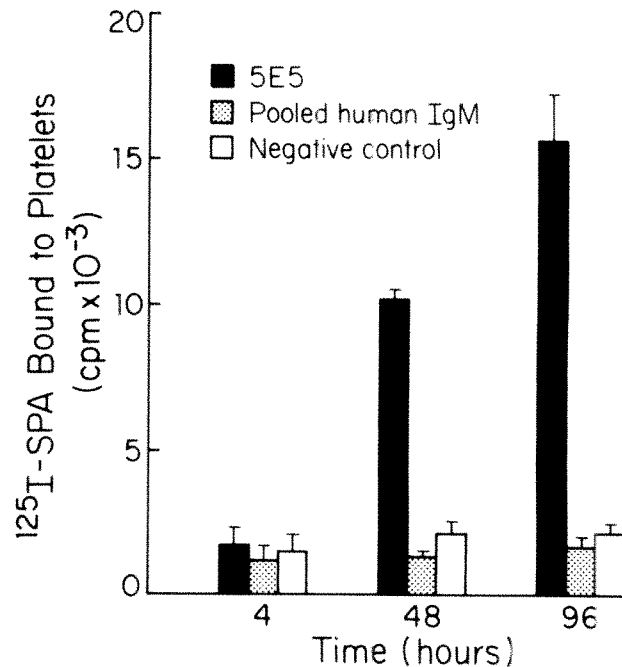


Fig 2. Indirect ^{125}I -staphylococcal Protein A platelet binding assay demonstrating reactivity of 5E5 as platelets age in vitro. The increase in expression of the epitope recognized by 5E5 is progressive as platelets age or become activated with storage, as compared with pooled human IgM or an isotype identical negative control human IgM monoclonal antibody. The results are mean values $\pm$ SD for five separate experiments.

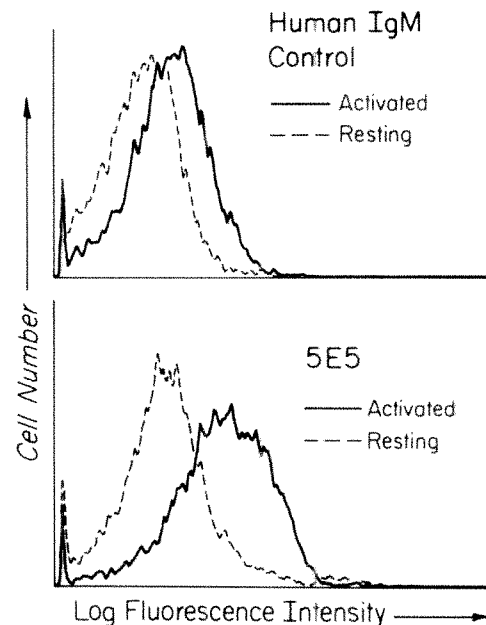


Fig 3. Indirect immunofluorescence analysis of antibody 5E5 binding to fresh resting v thrombin-activated platelets. As observed in stored platelets, a subset of thrombin-activated, fresh platelets (60%) demonstrates increased expression of the epitope recognized by 5E5 when compared with human IgM, the negative control antibody.

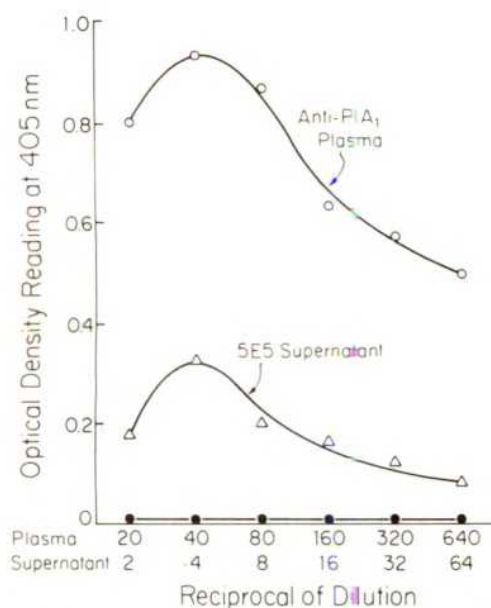


Fig 4. Enzyme-linked immunosorbent assay (ELISA) using purified GPIIb-IIIa adsorbed onto microtiter plates. Human plasmas specific for PIA¹ (○), control plasmas from normal individuals, pooled human IgM in tissue culture supernatant, six other platelet-reactive human monoclonal autoantibodies at similar concentration (●), and 5E5 supernatant (△) were tested on GPIIb-IIIa in triplicate. Only the 5E5 supernatants and human plasma with known anti-PIA¹ reactivity bound to the GPIIb-IIIa-coated plates. There was no reactivity of 5E5 or the PIA¹ positive plasmas on GPIb-coated plates (not shown).

whole platelet lysates was first separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose paper. 5E5 bound to a protein with an apparent mol wt of 95,000 under nonreduced conditions using lysates from either fresh or stored platelets, whereas other isotype-identical human monoclonal antibodies, at similar concentrations, did not bind to this protein in the identical lysate preparation (Fig 5). Plasma samples containing anti-PIA¹ antibodies known to react with GPIIIa were run simultaneously on the same immunoblot and bound to a platelet protein that comigrated with the protein recognized by 5E5.

In CIE studies, 5E5 reacted with the GPIIb-IIIa precipitin arc from both fresh and aged platelet preparations (Fig 6A). The control antibody, 3H8, did not bind to any of these precipitin arcs in lysates of either fresh or aged platelets (Fig 6B). In certain studies, 5E5 also bound strongly to a precipitin arc containing an as yet unidentified antigen that is often but not always present in lysates derived from stored platelets (Fig 6A). This precipitin arc was never observed in lysates prepared from fresh platelets.

DISCUSSION

We described the identification and preliminary characterization of a platelet antigen recognized by a human monoclonal autoantibody, 5E5, isolated from an ITP patient who, prior to this study, had never received any blood product. One explanation for the increased expression of the

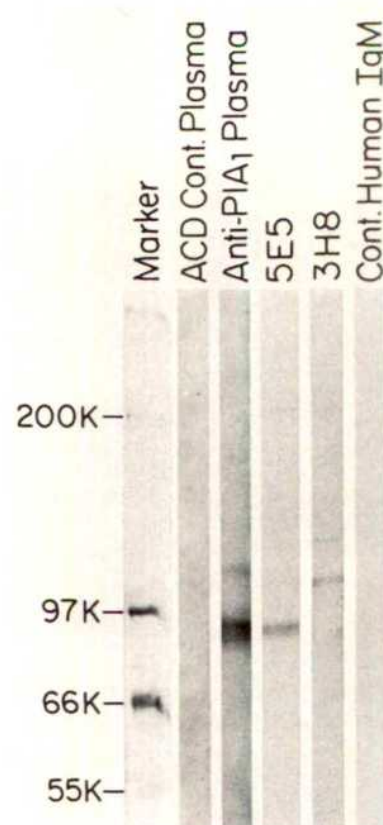


Fig 5. Immunoblot of platelet proteins. Under nonreducing conditions, 5E5 bound to a protein of 95,000 mol wt, as seen in lane 4. A nonplatelet binding human IgM monoclonal antibody isolated from an Epstein-Barr virus (EBV)-transformed cell line was also incubated with nitrocellulose strips as a negative control (lane 6). Antibody 3H8, a platelet-reactive human monoclonal autoantibody used in the crossed electroimmunophoresis studies, is shown in lane 5. Simultaneously, a normal plasma in lane 2 and a plasma containing anti-PIA¹ antibodies in lane 3 were screened for reactivity with immunoblotted platelet proteins. As is apparent in lane 3, the anti-PIA¹ antibodies reacted with a protein that comigrates with the protein recognized by 5E5.

5E5 epitope on GPIIIa is that platelets aged *in vitro* become activated during storage, resulting in specific membrane changes and the appearance of otherwise cryptic antigens on the cell surface. Rearrangements in the platelet membrane associated with activation, adhesion, or secretion are known to reveal neoantigens that might trigger immune-mediated clearance of these cells from the circulation.²¹⁻³² Increased levels of cell associated Ig resulting in shortened cell survival has been described for aged erythrocytes, monocytes, and platelets, as well as in disease states in which there is increased intravascular platelet activation.^{4-6,33,34,39} Therefore, it is reasonable to hypothesize that the expression of neoantigens as a result of senescence or activation leads to the binding of naturally occurring autoantibodies, accelerating clearance from the circulation.

In this study, we used platelet storage conditions that would currently be considered suboptimal, ie, storage in PL-146 containers on a rotary platform shaking apparatus.

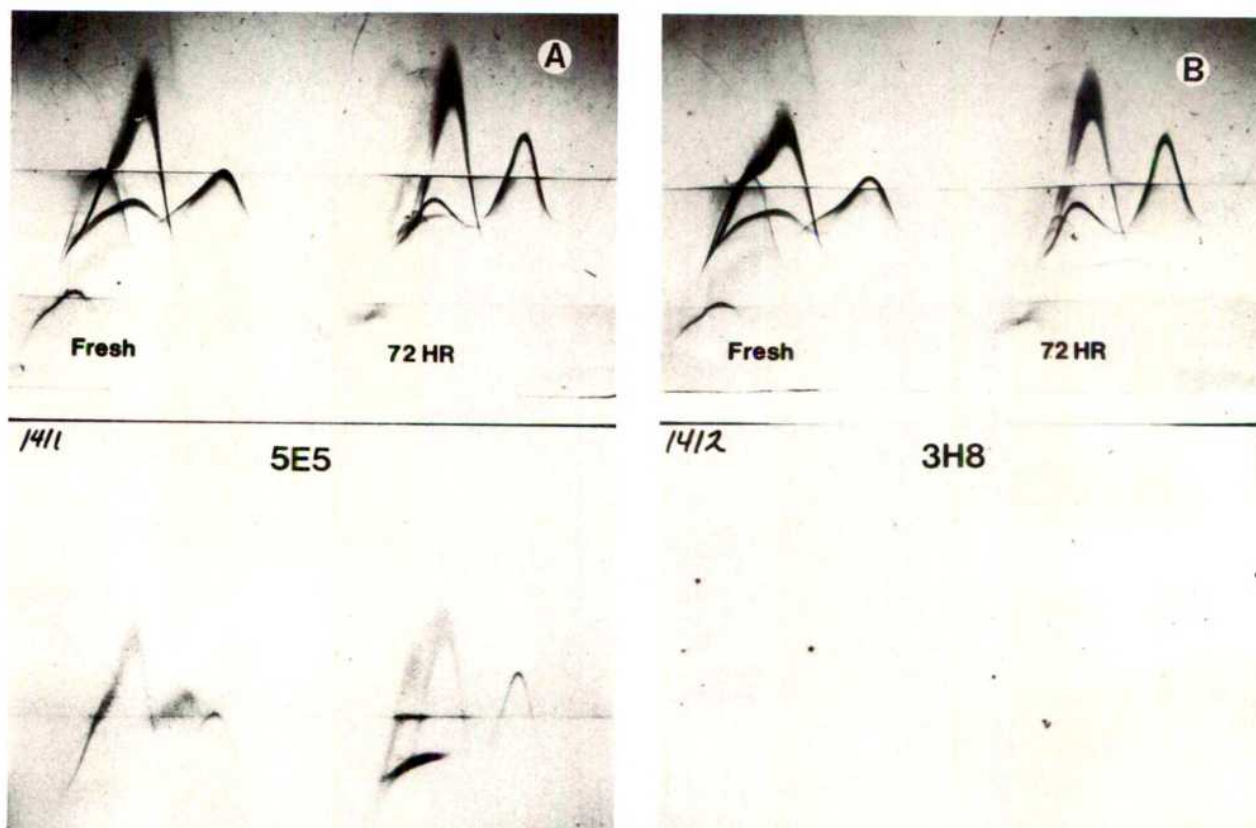


Fig 6. Crossed immunoelectrophoresis of platelet proteins. (A) The antibody 5E5 reacted with the GPIIb-IIIa precipitin arc from both fresh and aged platelet preparations. 5E5 also bound to an as-yet-unidentified precipitin arc present only in lysates prepared from stored platelets. (B) The control human antiplatelet antibody, 3H8, did not bind to any of these precipitin arcs in lysates of either fresh or aged platelets.

This decision reflects our intention to reproduce conditions that have been previously reported³⁵⁻³⁸ to result in altered platelet function and, perhaps, changes in platelet glycoprotein composition. In so doing, we hoped to mimic in vitro the effects of platelet aging in vivo. Use of optimal storage conditions, eg, PL-732 containers, and proper shaking apparatuses, would probably not result in significant platelet activation or glycoprotein changes. To demonstrate that this is so was not the thrust of the present study, however.

Isolation of senescence determinants from hematopoietic cells has thus far focused on erythrocyte antigens. Using antibody eluted from 50 L of aged erythrocytes, Kay³³ was able to immunoprecipitate a protein with an apparent mol wt of 62,000 that was believed to be related to band 3 of aged human erythrocytes. Using monospecific antibodies produced by rabbits immunized with this purified protein, Kay³⁴ identified polypeptides immunologically related to erythrocyte band 3 not only on other hematopoietic cell lines, such as mononuclear leukocytes and neutrophils, but also on nucleated somatic cells, such as human fibroblasts and squamous epithelium.

The unstimulated platelet undergoes an immediate shape change when exposed to specific agonists such as thrombin or ADP, resulting in the propagation of pseudopods and expression of receptors for circulating coagulation factors.^{22,23} Alterations in the association of GPIIb-IIIa with the platelet

cytoskeleton have also been reported following activation,²³⁻²⁶ and thrombin-induced secretion results in the expression of fibronectin, α granule membrane protein, and thrombospondin on the platelet surface.²⁷⁻³¹ In disease states associated with increased platelet destruction, increased quantities of plasma glycocalicin, a soluble glycopeptide derived from GPIb by the calcium-activated protease, calpain, have been demonstrated.³²

Similarly, aging or activation of platelets during storage under blood bank conditions results in both quantitative and qualitative changes in membrane glycoproteins. George³⁵ was among the first to report a decrease of $51\% \pm 4\%$ in membrane GPI in platelets which had been stored for 72 hours. These results were extended by the observations of White and colleagues,³⁶ who found a decrease in all major glycoproteins within 48 hours of storage. Furthermore, similar to our findings with in vitro aging, White described the appearance of several new polypeptides in stored platelets with electrophoretic mobilities corresponding to 260,000, 240,000, and 84,000. Bolin and colleagues³⁷ suggested that two additional glycoproteins, GP44 and GP77, which increase in expression as platelets age in vitro, may be proteolytic products of the GPIb molecule itself, since the appearance of these molecules on the platelet surface correlates statistically with a decrease in membrane GPIb. Alterations in platelet membrane glycoprotein composition may

account for the decreased adherence of stored platelets to subendothelium as determined by the Baumgartner perfusion technique.³⁸ These examples of membrane glycoprotein changes that occur during aging, activation, or damage represent just a few of the possible mechanisms for expression of new antigens that could target the platelet removal from the circulation.

The identification of platelet senescence or activation antigens using human autoantibodies reactive with these membrane proteins thus establishes a framework for further definition of the cellular and biochemical mechanisms

involved in the clearance of aged or damaged platelets in normal terminal maturation. Further studies are in progress to identify more precisely the nature and location of the epitope recognized by 5E5 on the GPIIIa molecule as well as to determine those conditions associated with increased expansion of the 5E5 epitope on platelets. The use of human monoclonal autoantibody from EBV-transformed B cell lines, rather than serum antibodies, provides a means to examine the expression of platelet antigens relevant not only to normal senescence of these cells but also to disease states characterized by increased platelet clearance.

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An Evaluation of Crossmatching, HLA, and ABO Matching for Platelet Transfusions to Refractory Patients

By Joanna M. Heal, Neil Blumberg, and Debra Masel

Refractoriness occurs in many patients receiving multiple platelet transfusions. We used a sensitive ELISA assay to assess the utility of crossmatching HLA-A,B matched single donor platelets in 51 consecutive, typical refractory patients. Of the 222 transfusions evaluated at 1 to 4 hours posttransfusion, only 17 of 54 (31%) with positive crossmatches had corrected platelet count increments of $\geq 7,500/\mu\text{L}$. In contrast, 95 of 168 (57%) of those with negative crossmatches had such increments ($P < .001$). Regardless of the results of the crossmatch, HLA-A,B, and ABO matching had independent influences on transfusion outcome. The median corrected 1- to 4-hour increment for crossmatch negative transfusions was 13,300/ μL for A/BU grade matches, 9,700 for BX, and 7,800 for C. Increments were 10,000/ μL for ABO-identical transfusions and 5,900 for transfusions of platelets ABO incompatible with the recipient's plasma antibodies. When the donor platelets were ABO compatible, but the donor plasma contained

ABO antibodies to the recipient's platelets, the increment was intermediate (8,200/ μL). The most important factor in predicting platelet survival was the crossmatch, followed by HLA-A,B and ABO, each having independent predictive value. These data demonstrate that the predictive value of a negative crossmatch may be considerably less than that reported in previous studies with stable, less ill patients. In typical refractory patients, there appear to be mechanisms of platelet destruction that are related to HLA-A,B and ABO but are not detected with current crossmatch methods. We hypothesize that soluble plasma HLA-A,B and ABO antigens contribute to the destruction of donor and sometimes recipient platelets by an immune complex or other "innocent bystander" mechanism. With our crossmatching technique, HLA-A,B and ABO match grades remain relevant to platelet transfusion therapy in some refractory patients.

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SINCE THE 1960s, platelet transfusion therapy has become vital to the prophylaxis and therapy of bleeding disorders in patients with leukemia, aplastic anemia, some lymphomas, and solid tumors.¹ The availability of effective platelet transfusion products has decreased mortality from bleeding in these patients.² Perhaps 50% of multiply transfused patients become clinically refractory.^{3,4} The causes of refractoriness to platelet transfusion appear to be primarily immunological,⁵ although factors other than alloimmunization may play an important role.⁶ HLA-A,B matching is one approach that has been fruitful in dealing with some refractory patients.⁷

Recently, questions have been raised as to whether refractoriness to platelet transfusion occurs purely on the basis of immunologic mechanisms.⁸ Such doubts are reasonable because the patients in question often have fever, infection, bleeding, coagulopathy, and splenomegaly. These nonimmunologic factors are known to decrease platelet recovery and survival. Sensitive and specific crossmatch techniques are now available for detection of IgG and IgM binding to donor platelets.⁹⁻¹¹ These tests appear to predict posttransfusion survival of platelets in patients with alloantibodies to donor platelets. However, many published studies excluded patients with the previously mentioned common factors that contribute to increased platelet consumption. Therefore, only a very small subset of refractory patients has been examined in detail.

We developed a sensitive enzyme-linked antiglobulin assay, and used it to evaluate platelet crossmatching retrospectively in typical refractory patients. We did not exclude any patients, because the utility of crossmatching in refractory patients preselected on the basis of alloimmunization has been clearly demonstrated.⁹ We reasoned that a study that does not exclude any patients would yield a realistic assessment of the potential benefits of crossmatching in typical clinical practice. It might also provide useful insights into the refractory state that could be missed if the study

were limited to patients who were alloimmunized as defined by lymphocytotoxic antibodies.

In addition, we analyzed the role of HLA-A,B and ABO matching because no analysis encompassing all these potentially important immunologic factors has been reported.

METHODS

Patient selection. Single donor platelets collected from a closely HLA-A,B matched, unrelated individual were crossmatched retrospectively against recipient plasma in 51 consecutive refractory patients for 316 transfusions. All patients had received RBC products from which leukocytes had not been removed, as well as numerous random donor platelet transfusions. All patients had had at least two previous transfusions of pooled random donor platelet concentrates that failed to yield satisfactory corrected platelet count increments ($\geq 7,500/\mu\text{L}$ at 1 to 4 hours). Lymphocytotoxicity screening for anti-HLA-A,B antibodies was not routinely performed, but all patients had detectable antiplatelet antibody by our enzyme-linked immunosorbent assay (ELISA) technique. Only one

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patient received granulocyte transfusions, concomitant with the platelet transfusions being evaluated by our crossmatch technique.

None of these patients was free of the "nonimmunologic factors" known to shorten platelet survival. That is, all had one or more of the following factors: fever $>99^{\circ}\text{F}$, sepsis or systemic infection, splenomegaly, consumption coagulopathy, bleeding, or a previous history of autoimmune thrombocytopenia. Determination of these factors was made by the physicians caring for the patient and was documented in the medical record. The presence or absence of these factors was not determined for each individual platelet transfusion.

The primary diagnoses were acute nonlymphocytic leukemia (21), solid tumor (5), acute lymphocytic leukemia (4), chronic myelogenous leukemia (3), preleukemia (3), aplastic anemia (2), lymphoma (2), and other hematological conditions such as myelofibrosis, erythroleukemia, multiple myeloma, etc. (11). Three of the patients were children aged <5 years. The other patients were adults. Three patients had undergone autologous or allogeneic bone marrow transplantation.

The HLA-A,B match grades of the platelet transfusions were A (7), BU (37), BX (71), and C (201). The data on the A and BU transfusions were pooled for analysis. Of the 51 patients, 25 received three or more evaluable transfusions. Of these, 16 received both B and C matches. Posttransfusion platelet count increments were measured in all patients, and the counts were corrected for body surface area and number of platelets transfused.⁹ Not all patients had increments measured at both 1 to 4 and 18 to 24 hours as planned; 222 transfusions had posttransfusion platelet counts done at 1 to 4 hours. The other 94 had the initial count done between 5 and 24 hours posttransfusion; 107 transfusions had counts performed at two posttransfusion time points.

Crossmatch technique. Immulon II microtiter plates (Dynatech, Chantilly, VA) are washed three times using distilled water. A murine monoclonal antibody that reacts with an undefined antigen present on all human platelets (UR 1-6.6 produced by the Laboratory Medicine Division Hybridoma Facility) is added to each well in the amount of 100 μL at a concentration of $\sim 10 \mu\text{g}/\text{mL}$ in coating buffer (1.59 g/L Na_2CO_3 , 2.93 g/L NaHCO_3 , pH 9.6). The plate is then incubated for 2 hours at 37°C and then overnight at 4°C . The antibody is used as crude mouse ascitic fluid. Platelets are first sedimented and resuspended in phosphate-buffered saline (PBS)-EDTA (0.01 mol/L of $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.15 NaCl, 3 g/L Na_2EDTA , pH 7.0) prior to adhering to the plate. After resuspension in PBS-EDTA at a count of 370,000/ μL , a 100- μL aliquot of platelets is added to each of two wells for each plasma to be tested. After platelets are added, the microtiter plate is centrifuged at 1,800 rpm in a GLC-4 centrifuge for 10 minutes to sediment the platelets onto the monoclonal antibody-coated surface. The plate is then incubated at room temperature for 50 minutes and is then washed semiautomatically, without need for centrifugation, three times using PBS (pH 7.2) and a Dynatech Mini-Wash. PBS-1% bovine serum albumin (BSA; BCA, West Chester, PA) 100 μL is added to each well to reduce nonspecific adsorption of plasma proteins and enzyme-antiglobulin conjugate during subsequent steps. This step requires a 30-minute room temperature incubation, and is followed by two further washes with PBS-1% BSA.

Test plasmas are diluted 1:2 in PBS-1% BSA to reduce nonspecific binding. Each diluted plasma (100 μL of each) is added to each of two wells and incubated for 45 minutes at 37°C if IgG is to be measured, or for 60 minutes at room temperature if IgM is to be measured. Appropriate positive (anti- PI^{A}) and negative plasma controls are also run in duplicate. The plate is washed three times using PBS-1% BSA, and 100 μL of alkaline phosphatase linked to affinity-purified anti-human IgG or IgM (Sigma Chemical, St Louis) is then added at a dilution previously optimized to the lot in question. The conjugates are usually used at 1:800 or 1:1,000

dilutions. Two wells receive no conjugate to serve as negative controls for nonspecific binding by the anti-IgG-enzyme conjugate. A further incubation at 37°C for 45 minutes (IgG) or room temperature for 60 minutes (IgM) is performed, followed by three PBS-1% BSA washes. Substrate, 1 mg/mL of *para*-nitrophenyl phosphate (Sigma) in 0.05 mol/L of Na_2CO_3 , 0.001 mol/L of MgCl_2 , pH 9.8 is added at 100 $\mu\text{L}/\text{well}$. Color development is measured spectrophotometrically¹² with a Dynatech MR600 microtiter plate reader coupled to an Apple IIe microcomputer for which kinetic-ELISA (k-ELISA) programs have been written by Dr Jonathan Cowles, Pittsford, NY; $\Delta\text{A}405 \text{ nm}/\text{min} \times 1,000$ is computed by least-squares linear regression of absorbance readings taken at 20, 25, 30, and 35 minutes after substrate addition. The correlation coefficient for the linearity of the $\Delta\text{A}405 \text{ nm}$ v time is usually 0.99, thus confirming the measurement of first-order enzyme kinetics, which we previously showed to correlate linearly with the amount of IgG and anti-IgG bound. The assay is linear throughout the range used.¹² The amount of IgG bound to the solid-phase platelet layer is reported as the mean of the duplicate determinations of $\Delta\text{A}405 \text{ nm}/\text{min} \times 1,000$. The duplicate values are usually within 10% to 20%. Because the kinetic measurement of enzyme activity is made at ambient temperature, controls are run to normalize the results from day to day.

The binding to platelets of IgG from plasmas of nonthrombocytopenic hospitalized patients yields a normal range of 0 to 1.08 for $\Delta\text{A}405 \text{ nm}/\text{min} \times 1,000$ (mean $\pm 2 \text{ SD}$, $n = 225$). The range for binding of IgM is 0 to 9.69 (mean $\pm \text{SD}$, $n = 122$). The positive control for the IgG assay is an anti- PI^{A} whose $\Delta\text{A}405 \text{ nm}/\text{min} \times 1,000$ is usually ~ 8.00 to 10.0 when run at a 1:1,000 dilution. The positive control for the IgM assay is a plasma from a patient with leukemia and autoimmune thrombocytopenia, which usually gives a $\Delta\text{A}405 \text{ nm}/\text{min} \times 1,000$ of 15 to 20 when tested at a dilution of 1:4. This latter plasma reacted with every donor platelet we tested. A positive crossmatch in both IgG and IgM assays was defined as binding $>2 \text{ SD}$ above the control means described above.

Titers of four randomly selected anti-HLA-A,B antibodies were 1 to 2 orders of magnitude higher with this k-ELISA technique than by lymphocytotoxicity assays. Titers of two anti- PI^{A} antibodies were 1 to 3 orders of magnitude greater by k-ELISA than by complement fixation techniques or ^{51}Cr release platelet cytotoxicity methods. Thus, we believe that the technique is probably of similar sensitivity to radioimmune⁹ or immunofluorescent¹¹ methods previously shown to be useful as platelet crossmatches. Although anti-A and -B blood group antibodies are detected by this assay, many patients nonetheless exhibit negative crossmatch results with ABO-incompatible platelets.

All patient plasmas were routinely tested (crossmatched) against four to six randomly selected normal donor platelets at the same time that the crossmatch was performed with the HLA-A,B matched platelets that were transfused. All patients had at least one positive crossmatch during the course of these tests, with almost all showing incompatibility with several non-HLA-A,B matched platelets. All HLA-A,B matched platelets for transfusion were also crossmatched with two to four normal plasmas. This approach allowed us to detect donor platelets that might have significantly elevated levels of IgG or IgM. Such platelets yield positive crossmatches even with normal donor plasmas. Donor platelets with elevated platelet-associated IgG (PAIgG) or IgM occurred very rarely and were excluded from the data reported.

Platelets were stored no more than 1 to 5 days at 4°C prior to crossmatching. Such storage does not alter the level of platelet surface IgG or IgM, or the binding of plasma IgG or IgM during a crossmatch (data not shown).

Measurement of PAIgG. PAIgG assay is a technical variant of that described for the detection of plasma anti-platelet antibody.¹³

PAIgG or soluble IgG standards compete with solid-phase IgG for binding to an alkaline phosphatase-linked anti-IgG antiglobulin reagent. The principle of the assay is that increased concentrations of soluble IgG or PAIgG will compete for anti-IgG and reduce anti-IgG binding to the solid-phase IgG. This assay can reproducibly detect changes of ~50 molecules per platelet of IgG.

Definitions of HLA and ABO match grades. For HLA, A matches are identical at all four HLA-A,B loci; BU matches are those sharing only two or three HLA-A,B antigens present on recipient cells yet possessing no HLA-A,B antigens that differ from those of the recipient due to being homozygous at one or both HLA-A,B loci; BX matches are those with one donor HLA-A,B antigen differing but cross-reactive with one of those of the recipient; C matches are those in which there is one non-cross-reactive antigen present on the donor cells that differs from those of the recipient. BUX matches were classified as BX.

For ABO, identical transfusions are those in which both donor and recipient are of the same ABO type (eg, an A donor to an A recipient); ABO "plasma-incompatible" transfusions are those in which the platelets do not carry antigens incompatible with recipient plasma, but in which the donor plasma contains antibody to ABO antigens possessed by the recipient (eg, an O to an A); in ABO "platelets-incompatible" transfusions, the donor platelets carry ABO antigens to which the recipient has antibody (eg, an A to an O).

Statistical analysis. All analyses were made using an Apple Macintosh microcomputer (Cupertino, CA) and NCSS software (Dr Jerry L. Hintze, Utah State University, Kaysville). Comparisons of medians of multiple groups were performed using nonparametric multivariate methods (Kruskal-Wallis). Medians were reported throughout the analysis because the data were not all normally distributed. The results of the analyses using parametric analyses that assume normally distributed data were identical to those reported. When statistically significant findings existed, individual comparisons of groups were made using the Mann-Whitney two-sample test, which is the nonparametric equivalent of the unpaired *t* test. Comparison of frequencies of events was performed using chi-square contingency tables, with individual frequencies being further compared to each other with Fisher's exact test. In addition, a three-way analysis of variance (ANOVA) was performed to assess the independent contributions of crossmatch results, HLA-A,B and ABO match grades to platelet count increments, and any interactions between these variables.

RESULTS

Of the 316 IgG crossmatches performed, 88 (28%) were positive. The frequency of positive crossmatches did not vary significantly with HLA match grade: A (14%), BU (28%), BX (28%), and C (30%). This conclusion applies only to BU, BX, and C matches because only seven A matches were studied.

The IgG crossmatch positivity rate was 33 of 129 for ABO identical transfusions (26%), 26 of 90 for ABO plasma-incompatible transfusions (29%), and 31 of 97 for donor ABO platelets-incompatible transfusions (32%). However, A donor platelets crossmatched with O recipients had the highest crossmatch positivity rate—23 of 54 (43%; $P < .02$ v ABO identical and $P < .07$ v ABO plasma-incompatible transfusions).

The IgM crossmatch positivity rate did not vary with HLA or ABO match grade. Of 308 IgM crossmatches performed, 41 (13%) were positive. However, in only 15

instances were the IgM crossmatches positive and the IgG negative. There was no clear correlation between posttransfusion corrected increment and crossmatch results in these few discrepant cases.

The success rate at 1 to 4 hours posttransfusion (a corrected platelet count increment $\geq 7,500/\mu\text{L}$) was 95 of 168 for crossmatch-negative transfusions (57%) and 18 of 54 for crossmatch-positive transfusions (33%) ($P < .001$). The success rate varied significantly with HLA and ABO match grade even for crossmatch-negative transfusions: A/BU (74%); BX (62%); C (51%)—ABO identical (66%); ABO plasma incompatible (54%); ABO platelets incompatible (43%). These differences were significant for A/BU v C ($P = 0.03$), and for ABO identical v ABO platelets incompatible ($P = 0.01$).

The median corrected platelet count increments arranged according to HLA match grade for crossmatch-negative transfusions are shown in Fig 1. A similar trend was observed for crossmatch-positive transfusions, but this was not statistically significant. Corresponding data arranged according to the degree of ABO match are shown for crossmatch-negative (Fig 2) and crossmatch-positive (Fig 3) transfusions. Regardless of the crossmatch results, ABO identical platelets gave significantly better results.

The role of HLA match grade was particularly notable when time periods ≥ 5 hours posttransfusion were examined (Fig 4). At these later time points, crossmatch-negative C match transfusions yielded increments in platelet count that were significantly inferior to those of A/BU and BX matches. The degree of ABO compatibility did not correlate significantly with posttransfusion increments at ≥ 5 hours posttransfusion, although a trend toward better results with ABO-identical platelets was observed.

To assess the relative roles of crossmatching, HLA matching, and ABO matching in predicting platelet count increments, two- and three-way ANOVAS were performed to assess the independent and interactional contributions of these factors. Crossmatching was the most significant predictive factor for corrected platelet count increments performed 1 to 4 hours posttransfusion ($P = .002$ v HLA-A,B and ABO). HLA matching ($P = .02$ v ABO) and ABO

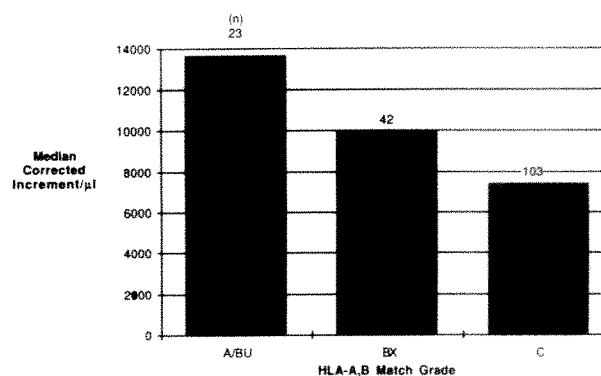


Fig 1. Crossmatch negative transfusions. Median corrected 1- to 4-hour posttransfusion platelet count increment plotted according to the HLA-A,B grade of the match. $P < .001$ for the three groups by Kruskal-Wallis. $P < .05$ for A/BU v C, $P = .16$ for A/BU v BX, and $P = .10$ for BX v C by Mann-Whitney.

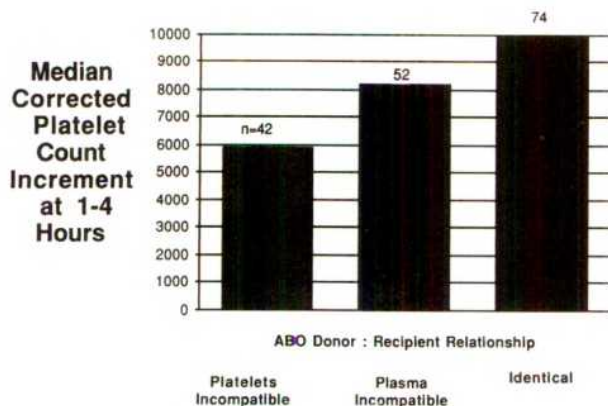


Fig 2. Crossmatch negative transfusions. Median corrected 1- to 4-hour posttransfusion platelet count increment plotted according to the ABO relationship between donor and recipient. The Kruskal-Wallis analysis yielded a significant difference among the groups ($P < .05$). $P = .07$ for platelets ABO identical v platelets ABO incompatible by Mann-Whitney. When grouped according to HLA-A,B match grade, the difference between platelets ABO identical and platelets ABO incompatible yielded $P = .12$ for C matches, $P = .03$ for BX matches, and $P = .15$ for A/BU matches. The differences between plasma ABO incompatible and the other two groups were not statistically significant ($P = .16$ v identical; $P = .83$ v platelets incompatible). When the two groups of ABO nonidentical platelets were merged and compared with the ABO-identical platelets group, the difference was significant ($P = .048$).

matching ($P = .08$ v crossmatching) had independent effects to a lesser degree. None of the interaction terms in these analyses was significant, confirming the largely independent roles of these three factors.

When the transfusions are grouped by crossmatch results, HLA-A,B match grade and ABO match grade, and then ranked by median corrected platelet count increment performed 1 to 4 hours posttransfusion, the independent and significant roles of these factors are clear (Table 1). Favor-

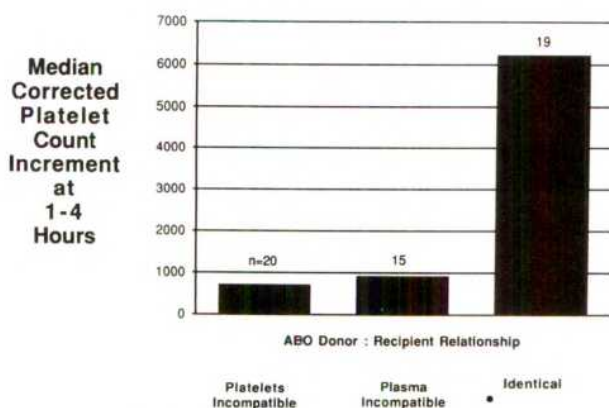


Fig 3. Crossmatch positive transfusions. Median corrected 1- to 4-hour posttransfusion platelet count increment plotted according to the ABO relationship between donor and recipient. The Kruskal-Wallis analysis did not yield a significant difference among the groups ($P = .19$). When the two groups of ABO nonidentical platelets were merged and compared with the ABO-identical platelets group, the difference in the medians by Mann-Whitney test was significant at $P = .07$.

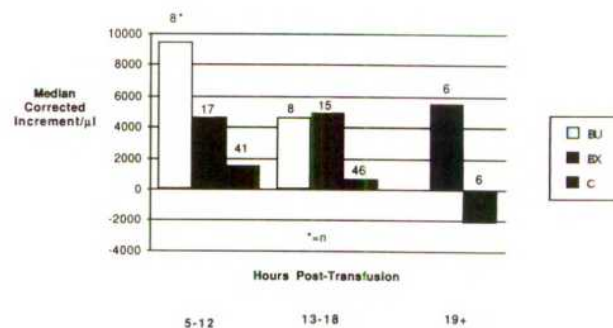


Fig 4. Crossmatch negative transfusions. The median corrected posttransfusion platelet count increment at times ≥ 5 hours posttransfusion according to HLA-A,B match grade. BU includes A matches as well. By Kruskal-Wallis, the differences between A/BU and BX were not significant at any point, and the differences between C matches and both A/BU and BX matches were significant at all time points ($P < .05$ by Kruskal-Wallis for 5 to 12 and 13 to 18 hours; $P < .05$ by Mann-Whitney for BX v C at ≥ 19 hours posttransfusion).

able HLA and ABO match grades can apparently compensate for crossmatch incompatibility in some instances. For example, the small number of crossmatch-positive A, BU and BX transfusions that were also ABO identical did as well or better than crossmatch-negative C matches that were ABO platelets incompatible. Similarly, ABO matching can compensate for poorer HLA-A,B matching. Crossmatch-negative C matches that were ABO identical did as well or better than crossmatch-negative BX matches that were ABO nonidentical.

An unexpected finding was that after crossmatch-positive transfusions, the platelet count at 1 to 4 hours posttransfusion was frequently lower than the pretransfusion platelet count. Fifteen of 54 crossmatch-positive transfusions (27%) led to platelet count decrements as compared with only 14 of 168 (8%) for crossmatch-negative transfusions ($P < .0005$). A separate analysis of this phenomenon will be reported elsewhere.

To assess whether the decreased survival of transfused platelets in patients receiving crossmatch-negative transfusions might have an immunological basis, we measured PAIgG in 18 of our refractory patients at various times during the period when transfusions were given. All the refractory patients, including those receiving predominately crossmatch-negative platelets, had elevated PAIgG at the levels seen in autoimmune thrombocytopenia (Fig 5). However, none of these patients had received exclusively crossmatch-negative, HLA-A,B-identical, and ABO-identical platelet transfusions. Elevations of PAIgG were noted after transfusions of both crossmatch-positive and crossmatch-negative platelets, as well as prior to transfusion with HLA-A,B-matched platelets.

DISCUSSION

Our study represents the first attempt to evaluate in one group of patients the three known immunologic factors that affect the success of platelet transfusions to refractory patients—crossmatching, HLA-A,B matching, and ABO

Table 1. Corrected Platelet CI According to Crossmatch Results, HLA-A,B and ABO Match Grades: Ranked by Median Corrected Increment

CI	X-M	HLA*	ABO	n	Success Rate (% CI $\geq 7,500/\mu\text{L}$)
17,700	Neg	BU	Platelets incompatible	7	86
17,000	Neg	BU	Plasma incompatible	7	71
11,400	Neg	BX	Identical	22	73
11,300	Neg	BU	Identical	9	67
11,100	Pos	BU/BX*	Identical	9	56
10,400	Neg	BX	Plasma incompatible	11	82
9,300	Neg	C	Identical	43	63
5,100	Neg	BX	Platelets incompatible	9	11
5,100	Pos	C	Identical	10	40
4,500	Neg	C	Platelets incompatible	26	42
4,300	Neg	C	Plasma incompatible	34	41
3,300	Pos	BU/BX	Plasma incompatible	2	0
1,000	Pos	C	Platelets incompatible	14	29
900	Pos	C	Plasma incompatible	13	23
200	Pos	BU/BX	Platelets incompatible	6	17

NOTE. The median category is an increment of 5,100/ μL . Of the crossmatch negative groups, seven fell at or above the median, only two fell below. Of the BU or BX match groups, seven fell at or above the median category, only two fell below. Of the five ABO-identical groups, all five fell at or above the median, none fell below. Conversely, most of the crossmatch-positive or C-match or ABO-platelet incompatible groups fell below the median group.

Abbreviation: CI, count increment.

*Only four A matches were included in these data and are grouped with the BU matches. Because so few BU and BX matches were crossmatch positive, these data were pooled for positive crossmatches.

matching. On initial examination, our results showing relatively poor predictive performance of a sensitive crossmatch may appear to conflict with optimistic prior reports.^{9,11,14-17} However, in some previous crossmatching studies patients were selected to be free of sepsis, severe bleeding, consumption coagulopathy, and splenomegaly.¹⁴⁻¹⁷ In our study and others,^{8,18-21} all patients were included. Our results are very similar to those reported in studies in which no patients were excluded (Table 2). All studies found positive predictive values (percentage of failures after a positive crossmatch) that were in the range of 70% to 100%. However, the negative predictive value (percentage of successes when the crossmatch was negative) was consistently higher (80% to 92%) in studies with preselected patients,¹⁴⁻¹⁷ and consistently lower (52% to 77%) when all patients were included.^{8,18-21} These lower success rates in typical refractory patients occurred regardless of crossmatch methodology used, including sensitive techniques such as radioimmunoassay.

"Nonimmunologic" factors such as infection, consumption coagulopathy, fever, bleeding, and splenomegaly may have accounted for the fact that 43% of our crossmatch negative transfusions yielded unsatisfactory increments. Inconsistent with this interpretation is the observation that the 45 crossmatch-negative transfusions of BU or BX match grade yielded a failure rate of only 26% (top four lines in Table 1). This failure rate of crossmatch-negative, closely HLA-A,B matched transfusions compares well with the

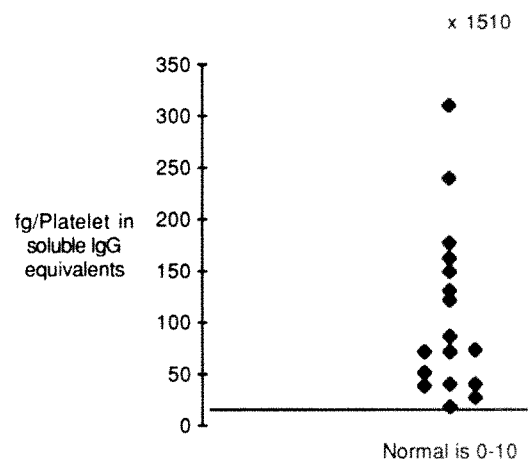


Fig 5. The platelet-associated IgG (PAIgG) in 18 refractory patients, measured by a competitive enzyme-linked immunosorbent assay (ELISA) technique is shown. The amounts are in femtograms (fg) of soluble IgG equivalents per platelet.¹³ None of the patients had normal values, including those who had received predominately crossmatch negative transfusions. These levels were generally measured at the nadir of the patient's platelet count, although all patients had received multiple RBC and platelet transfusions prior to testing. Some had also recently received pooled random donor platelets. Approximately 5% to 10% of nonthrombocytopenic hospitalized patients have elevated levels of PAIgG, predominately in the range of 10 to 20 fg/platelet.

overall failure rates reported in studies in which only stable, selected patients were reported: 8%, 20%, 8%, and 13%.¹⁴⁻¹⁷ Thus, nonimmunologic factors unrelated to crossmatch, HLA-A,B and ABO may account for at most ~10% to 20% of transfusion failures in typical refractory patients. An exact estimate of the role of nonimmunologic factors in platelet transfusion failures is not possible from our data because we did not examine in detail the clinical factors that might cause unsatisfactory increments in crossmatch-negative transfusions.

Our crossmatch technique appears to be sensitive to HLA-A,B, ABO and platelet-specific antibodies such as anti-PIA¹. Therefore, it is surprising that for crossmatch-negative transfusions, HLA-A,B and ABO matching still played a significant role in predicting transfusion success. Negative crossmatches led to corrected increments of $\geq 7,500/\mu\text{L}$ at 1 to 4 hours posttransfusion in only 57% of cases. HLA-A,B matching alone yielded a 50% success rate. The simplest possible explanation would be that our crossmatch technique is not sufficiently sensitive to HLA-A,B antibodies. We believe that this is unlikely based on its ability to detect increases in platelet-bound IgG at the level of 50 to 100 IgG molecules per platelet,¹³ and our success in detecting HLA-A,B antibodies detected by lymphocytotoxicity techniques. However, we have not made an exhaustive study of this aspect of our assay. If our platelet crossmatch assay is insensitive to some HLA-A,B antibodies, this is also likely to be true of other published assays. Other laboratories using different methodologies but similar patient selection criteria have also found low success rates for crossmatch-negative HLA-A,B matched transfusions: 52% to 77% (Table 2).^{8,18-21} It is possible that clinically significant types of

Table 2. Comparison of Some Recent Platelet Crossmatch Studies

Patients	n	Positive Crossmatches (%)	Negative Crossmatches (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	Crossmatch Method
Selected*						
Freedman ¹⁴	47	45	55	85	92	RIA
Ware ¹⁵	68	54	46	89	80	RIA
Kickler ¹⁶	230	40	60	73	92	RIA
Yam ¹⁷	48	38	62	87	87	RIA
Unselected						
Kakaiya ¹⁸	43	37	63	75	52	ELISA
Rachel ¹⁹	80	25	75	100	63	RBC-IA
Nusbacher ²⁰	67	33	67	100	77	RIA
Oldfather ²¹	254	NR	NR	98	63	LCT-AHG
McFarland ⁸	289	NR	NR	NR	67	RIA
Heal (present study)	222	24	76	67	57	ELISA

NOTE. Comparison of results of studies in which the patients were "selected" to be free of factors known to compromise platelet recovery and survival after transfusion, such as sepsis, fever, bleeding, consumption coagulopathy, autoimmune thrombocytopenia, and splenomegaly, and studies in which all refractory patients were studied, without exclusions (ie, "unselected").

n, number of crossmatches performed; Positive (%), percentage of all crossmatches positive; Negative (%), percentage of all crossmatches negative; positive predictive value, percentage of transfusions with positive crossmatches that had unsatisfactory platelet count increments as defined by the authors; negative predictive value, percentage of transfusions with negative crossmatches that had satisfactory increments as defined by the authors; crossmatch method, primary serological technique used as crossmatch in the study.

Abbreviations: RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; RBC-IA, RBC immune adherence; LCT-AHG, anti-human globulin-augmented lymphocytotoxicity; NR, not reported.

anti-platelet antibody are not readily detected by any of the existing techniques, including our own. Further study of this possibility is needed before definitive conclusions can be drawn about the role of crossmatching in refractory patients.

Transfusions of donor plasma containing ABO antibodies incompatible with the recipient's ABO antigens were associated with poorer platelet count increments in our study. Even in crossmatch-positive transfusions, HLA-A,B and ABO matching appeared to contribute to predicting post-transfusion platelet count increments. These findings cannot be explained by the insensitivity of the crossmatch used. Thus, the relatively disappointing performance of platelet crossmatching in our study and some of those previously reported^{8,18-21} are not due solely to nonimmunologic factors affecting platelet destruction or to the insensitivity of the crossmatch technique used. We suspect that the "false-negative crossmatches" are due to multiple factors in typical refractory patients.

One factor in a few instances might have been the dosage of platelets administered. A standard single donor platelet transfusion may be expected to raise the platelet count by 25,000 to 60,000/ μ L in a patient who is thrombocytopenic and without other reasons for decreased platelet recovery and survival. This corresponds to a corrected increment of 10,000 to 20,000/ μ L in a 1.7-kg adult given 4×10^{11} platelets. Thus, if platelet consumption is as little as 1.3 to 3 times normal due to splenomegaly, infection, bleeding, consumption coagulopathy, or a combination of these, the corrected increment might be $\leq 7,500/\mu$ L solely on the basis of inadequate dosage. Unfortunately, increasing the dosage of HLA-A,B matched single-donor platelets is both logistically difficult and extremely expensive. The importance of dosage is sup-

ported by our observation that 6 of the 15 satisfactory increments observed despite positive platelet crossmatches occurred in very small children receiving full adult dosages of platelets. Baldini and colleagues reported that giving very large dosages of incompatible platelets to children unresponsive to platelet transfusion could temporarily overcome their refractoriness.²²

A second possible explanation for false-negative crossmatches is that immunological causes of refractoriness exist other than antibody specific for platelet surface antigens. If this is so, it should not be surprising that in vitro assays such as crossmatching fail to predict in vivo results consistently. Indeed, our data show that in vivo findings, such as post-transfusion platelet count increments and level of IgG on circulating platelets, are not fully predicted by the results of crossmatching. Thus, additional hypotheses for immunologic mechanisms of platelet destruction in typical refractory patients are in order.

The additional causes of platelet destruction in refractory patients, not detectable by current crossmatch techniques, are probably immunologic. We base this conclusion on the important role that HLA-A,B matching and ABO matching appear to play in transfusions without detectable antiplatelet antibody. Also, our refractory patients universally had platelets coated with large quantities of IgG. This increased IgG may be due to previous incompatible platelet transfusions, as none of our patients received only crossmatch-negative, HLA-identical, and ABO-identical transfusions. Elevations in PAIgG were noted prior to transfusion and after both crossmatch-positive and crossmatch-negative transfusions. Thus, this IgG may be a nonspecific finding of no clinical significance. This increased IgG may also be due to immunologic mechanisms other than platelet-specific antibody

detectable in a crossmatch. Such immunologic mechanisms could be characteristic of the refractory state.

Two possibly relevant mechanisms are suggested by previous investigations of immune destruction of platelets due to drugs and microorganisms. Shulman and Jordan²³ and, more recently, Christie and Aster²⁴ demonstrated that platelets can acquire drug and IgG complexes by at least two pathways. One is the binding of IgG-drug complexes to platelet Fc receptors. The other is the binding of IgG, drug, and platelet in a complex that requires all three for antibody fixation on the platelet. Kelton and associates showed that the thrombocytopenia of malaria is due to acquisition of malarial antigens and the corresponding IgG antibody by the patient's platelets.²⁵ We propose that similar innocent bystander mechanisms involving soluble donor plasma HLA-A,B and ABO antigens may exist. This hypothesis is supported by our finding that, independent of the crossmatch result, HLA-A,B and ABO matching predict the posttransfusion platelet count increment. Platelets can acquire new blood group antigens such as HLA-A,B^{26,27} and ABO^{28,29} from plasma. Transfused plasma ABO and HLA-A,B antigens may affect platelet recovery and survival even when the platelet surface versions of these antigens are not detected in the crossmatch.

Paradoxically, transfusions of ABO-compatible platelets that are accompanied by ABO-incompatible plasma yield poor platelet count increments (eg, O platelets to an A recipient). This new finding requires confirmation. One hypothetical explanation for these results is that anti-A from the donor plasma combines with soluble A antigen in the recipient's plasma, and then damages the type O donor platelets by an immune-complex mechanism. Alternatively, the type O transfused platelets may acquire A antigen in the recipient's plasma and subsequently be injured directly by the anti-A from the donor. If such mechanisms exist, incompatible transfusions might affect the fate of subsequent transfusions that were otherwise immunologically compatible. Any role for plasma HLA-A,B and ABO antigens in platelet transfusion immunology remains speculative and will require further investigation.

If our preliminary results are confirmed by other researchers, the role of platelet crossmatching in typical refractory patients clearly may be more limited than has been expected. We and others had hoped that crossmatching would make HLA-A,B matching unnecessary and enable the use of

randomly selected crossmatch-negative pools of donor platelets. Our data do not provide much cause for optimism in this regard, at least in typical refractory patients. In our patients, crossmatch-negative C-matched platelets do reasonably well in the first few hours posttransfusion. However, later C-matched platelets appear to be considerably less likely to provide satisfactory platelet count increments than do A-, BU-, or BX-matched platelets. Some of this disadvantage may be overcome by using ABO-identical C-matched platelets. Our findings support a role for ABO in platelet transfusions, first studied by Aster ~20 years ago.³⁰ New data on possible roles for ABO³¹ and HLA-A,B³² matching have recently been reported by other investigators.

Our study has certain practical implications for changing the manner in which platelet donors are selected for refractory patients. If an IgG crossmatch technique is available and practical, it should be used to avoid transfusing donor platelets that are crossmatch positive with the recipient. Closely HLA-A,B-matched platelets should be given next priority. If C or worse grades of match are the only ones available, ABO identical donors should be selected if feasible. Our data suggest that an IgM crossmatch will not add significantly to the clinical benefit to be gained by crossmatching in most cases.

The alleviation of refractoriness in many patients may be quite difficult, if our results are typical. Further research into the multiple mechanisms of immunologic refractoriness to platelet transfusion is indicated, and efforts to prevent refractoriness^{33,34} warrant additional attention.

NOTE ADDED IN PROOF

Brand et al recently reported ABO antibodies as a cause of platelet refractoriness (*Transfusion* 26:463, 1986).

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Identification of a Defective Factor XI Cross-Reacting Material in a Factor XI-Deficient Patient

By C. Mannhalter, P. Hellstern, and E. Deutsch

A homozygous factor XI-deficient girl, who appeared to be positive for cross-reacting material (CRM⁺) was studied for clarification. Factor XI antigen (F XI:Ag) was measured by radial immunodiffusion using monospecific, heterologous anti-factor XI antibodies. Factor XI coagulant activity (F XI:C) was determined in a modified activated partial thromboplastin time (APTT) test. The ratio of F XI:C to F XI:Ag was 0.04 for the proposita, as compared with 0.7 to 0.74 in the other family members. In contrast, 12 normal individuals had ratios of F XI:C to F XI:Ag of 1.04 ± 0.15 . F

FACTOR XI (plasma thromboplastin antecedent) deficiency is a rare hereditary blood coagulation disorder. Since the first report by Rosenthal in 1953,¹ several hundred cases have been recognized, most of them among Ashkenazi Jews.² Only a few well-documented cases have been observed in persons of non-Jewish origin.³⁻⁶ All publications to date agree that hereditary factor XI deficiency represents a genetic disorder and is caused by the absence of the molecule.⁷⁻⁹ Genetic heterogeneity of hereditary factor XI deficiency has been suspected, but has not yet been established. However, it deserves particular consideration, since factor XI-deficient patients differ greatly in their bleeding tendency.^{10,11} This article reports the studies of a patient with normal levels of factor XI antigen (cross-reacting material, CRM) and severely reduced clotting activity not due to a circulating antibody. The data elucidate the kind of this factor XI defect.

MATERIALS AND METHODS

All chemicals obtained from commercial sources were the best grade available. Micronized kaolin was generously supplied by Helena Laboratories, Beaumont, TX. Soybean trypsin inhibitor (SBTI) was purchased from Serva, Heidelberg, FRG. Corn trypsin inhibitor (CTI) was prepared according to the methods of Kirby and colleagues.¹² The protein concentration of our preparation was 1.5 mg/mL as determined by the Lowry method.¹³ The chromogenic substrate S-2366 was a generous gift of Kabi Vitrum, Stockholm. Radial immunodiffusion experiments were carried out with Seakem agarose from FMC, Rockland, ME. Anti-F XI antibodies were either a gift of Dr B.N. Bouma, Utrecht, Holland, or were obtained from Nordic, Tilburg, Holland. Anti-F XII antibodies were from Behring-Werke, Marburg, FRG. All reagents for polyacrylamide gel electrophoresis were purchased from Bio Rad, Richmond, CA. Nitrocellulose membrane (Hybond C) was obtained from Amersham, Buckinghamshire, UK. For autoradiography, Kodak S X-ray films and sapphire intensifying screens were used at -70°C .

Patients' Blood Samples

Samples were drawn from the antecubital vein using a 19-gauge butterfly needle. Nine parts blood were mixed with 1 part 0.1 mol/L of sodium citrate. Platelet-poor plasmas (PPPs) were obtained by 20-minute centrifugation at 2,000 g. The plasmas were aliquoted into 0.3-mL aliquots and stored at -20°C .

Coagulation Activities

Coagulation activities were assayed in a modified activated partial thromboplastin time (APTT) system using hereditary deficient

XI esterolytic activity was clearly higher than F XI:C in the proband, but not in her relatives. Immunoblotting studies demonstrated F XI CRM in the patient's plasma. Chromatography on diethylaminoethanol (DEAE)-Sephadex at pH 8.4 led to an almost complete removal of F XI from the plasma. The defective F XI was not bound to a negatively charged kaolin surface due to an abnormal interaction with high-mol-wt kininogen (HMWK).

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plasmas as described previously.¹⁴ F XI- and F XII-deficient plasmas were obtained from Behring-Werke, prekallikrein (PK) and high-mol-wt kininogen (HMWK)-deficient plasmas were purchased from George King Bio-Medical, Overland Park, KS. If not mentioned otherwise, kaolin (Behring-Werke, 20 mg/mL of saline) was the activating surface. In selected experiments, ellagic acid (Actin; Merz and Dade, Düringen, Switzerland) was used as activating surface in the clotting assays following the manufacturer's instructions.

Esterolytic Activity of Factor XI

Esterolytic activity of factor XI was determined according to Scott and associates.¹⁵ In brief, plasma dilutions were treated with CHCl_3 to remove C1-esterase inhibitor. Activation of F XI was achieved with micronized kaolin (final concentration in the assay 0.5 mg/mL) in the presence of SBTI (5 $\mu\text{mol/L}$). After 30-minute incubation at 37°C , F XIIa was inhibited with CTI (final concentration 15 $\mu\text{g/mL}$). The esterolytic F XIa activity was quantitated at 405 nm by measurement of the cleavage of the chromogenic substrate S-2366 at 37°C . All plasma dilutions were made with F XI deficient plasma (Behring).

Antigen Concentrations

Antigen concentrations of F XI and F XII were measured by radial immunodiffusion (RID). The F XII antibody was used at 2%; the F XI antibody was used at 1.5% in 1% agarose. The incubation time needed to form distinct F XI-anti F XI precipitates was 24 hours at room temperature and 6 days at 4°C . The F XII plates were incubated for 24 hours at room temperature and 3 days at 4°C . After extensive washing, the immunoprecipitates were stained with Coomassie brilliant blue.

Kaolin-Binding Studies

For kaolin-binding studies, undiluted plasma samples were incubated with kaolin or micronized kaolin (final concentration 2.8 mg/mL) for 5 minutes at 37°C with repeated mixing. After centrifugation

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gation in a Beckman Microfuge B, the supernatants were tested for factor XI:Ag by RID. Control samples containing saline instead of kaolin were treated identically. RID was performed as described above.

Effect of Patient Plasma on Normal Plasma Factor XI Activity

We tested the presence of a factor XI inhibitor in the patient's plasma by incubation of a constant amount of patient plasma with varying amounts of normal plasma for 60 minutes at 37°C, followed by a determination of the F XI clotting activity.

Purification and Radiolabeling of F XI

F XI was purified from human plasma by sequential chromatography as described previously.¹⁹ The purified protein had a specific activity of 138 U/mg. This F XI was radiolabeled with immobilized lactoperoxidase (Radioiodination System; New England Nuclear, Dreieich, FRG) according to the manufacturer's instructions.

Electrophoretic Studies and Western Blot Analysis of Normal and Patient's Plasma Samples and Pretreated Plasmas

Pretreatment of the plasmas with diethylaminoethanol (DEAE)-Sephadex. Normal citrated plasma as well as the proposita's plasma were treated identically in the following ways: Plasma aliquots (50 mL) were dialyzed against 1 L Tris-succinic acid buffer (0.04 mol/L of Tris, 0.01 mol/L of succinic acid, 0.001 mol/L of EDTA, 0.001 mol/L of benzamidine, 0.02% sodium azide, 50 mg polybrene/L, pH 8.4) at room temperature overnight with two buffer changes. The dialyzed samples were applied to a DEAE-Sephadex column (2.5 × 37 cm) equilibrated with the same buffer. In normal plasma, the elution of factor XI from the column was monitored by determination of the factor XI clotting activity. F XI was found in the first protein peak passing through the column. In the patient's plasma, which lacked F XI:C, this first protein peak was pooled analog to normal plasma and used for further studies.

Kaolin Adsorption of the Plasmas. Aliquots of normal plasma, the proposita's plasma and a factor XI-deficient patient's plasma (200 µL each) were incubated with 32 µL of kaolin suspension (20 mg/mL of saline) for 5 minutes at 37°C. The samples were then spun for 1 minute in a Beckman Microfuge, washed with three times 1 mL of saline and eluted overnight with 120 µL 6% sodium dodecyl sulfate (SDS) in stacking gel buffer. After spinning for 1 minute in the Microfuge, 60 µL of each supernatant was applied to SDS-polyacrylamide gel electrophoresis (PAGE).

Electrophoretic separation on SDS gels. Samples were mixed with appropriate amounts of stacking gel buffer containing 6% SDS and treated for 3 minutes in a boiling water bath. The samples were separated on 7.5% SDS-polyacrylamide slab gels (1.5 mm thick) according to the method of Laemmli.¹⁷ Separation was carried out using constant current (35 mA/gel) and tap water cooling.

Blotting conditions. After electrophoresis, gels were immediately used for electroblotting onto nitrocellulose (Hybond C, Amersham) according to the general method of Towbin and colleagues.¹⁸ Proteins were electrotransferred at 1.5 V/cm overnight or 5 V/cm for 2 hours in a Tris-glycine buffer (25 mmol/L of Tris–190 mmol/L of glycine pH 8.3) at 10°C using a Bio Rad Trans-Blot system. After transfer, the nitrocellulose membranes were quenched with 0.4% gelatin–0.2% Tween–0.02% NaN₃ in phosphate-buffered saline (PBS) pH 7.2 for 4 hours. This solution was used in the following steps as medium for the antibody and the labeled F XI. The washing solution used throughout the procedure was PBS containing 0.2% Tween and 0.02% NaN₃. Immunodetection of the antigens blotted

onto nitrocellulose was achieved by interaction with an immunoadsorbed polyclonal goat–anti-human factor XI antibody for 16 hours. After three washes (15 minutes each), the bound factor XI antibody was traced with ¹²⁵I-factor XI (incubation for 4 hours), followed by three more washes (15 minutes each). All incubations were done at room temperature. The washed membranes were dried and set up for autoradiography at –70°C.

Immunoabsorption of goat–anti-human F XI antibody. A sheet of nitrocellulose (15 × 15 cm) was incubated with F XI-deficient plasma (1 mL in 25 mL of quenching buffer) for 4 hours at room temperature. The membrane was then quenched with gelatin as described above. One such sheet was used for immunoabsorption of 50 mL of a 2% antibody solution in quenching buffer.

Clinical information. A more detailed description of the patient is given in a previous article.¹⁹ In brief, the patient, a 12-year-old girl (G.S.) suffered from a lifelong tendency to develop large hematomas after trivial traumata. Severe bleeding occurred after dental extraction and after adenectomy. Her mother (G.K.) and her sister (G.E., aged 14 years) also experienced bleeding episodes, whereas the father (G.M.) did not. Jewish ancestry and consanguinity between the parents could be ruled out. Coagulation tests for F VIII and F IX, measurements of F VIII:Ag, F VIII:R:CoF, platelet adhesiveness, platelet aggregation, and template bleeding time were normal, as reported previously.¹⁹

RESULTS

Evaluation of Contact Phase Factors by Coagulation Assays

The clotting assays of the four contact factors at the time of the investigation exhibited normal activities of prekallikrein (PK) and high-mol-wt kininogen (HMWK) in all family members studied. The factor XI levels in the patient's plasma and her relatives' plasma had been evaluated at different times in Germany as well as in Austria. Factor XI:C data were obtained in 1984 in both laboratories from the same plasma source, and they correlated very well. They reflected reduced F XI levels in the mother and the proposita, and borderline F XI concentrations in the father (Table 1). However, when fresh blood samples were collected in 1985 and were assayed with F XI-deficient plasma (Behring) we obtained the following results. The factor XI activity in the proposita's and her father's plasma was similar to the first investigation. The mother's and the sister's plasma exhibited higher factor XI levels, within the normal range (Table 1). Interestingly, the determination of F XI:C with a different deficient plasma (Immuno) in the proposita's, her mother's, and her sister's plasma gave clearly lower F XI:C values (Table 2).

Table 1. Factor XI:C Data 1984 and 1985

Subject	1984 (U/mL)	1985 (U/mL)
Father	0.63	0.69
Mother	0.49	0.64
Sister	—	0.63
Proposita	0.02	0.04
Normal range	0.6–1.4	

Data for 1984 represent mean values of two different coagulation assays of blood samples. Data for 1985 represent mean values of two independent coagulation assays of blood samples analyzed with factor XI-deficient plasma, Behring.

Table 2. Clotting Activities and Antigen Concentrations of Contact Factors in Patients and Controls

Parameter (U/mL)	Proposita G.S.	Sister G.E.	Mother G.K.	Father G.M.	Normal Range
F XI:C (F XI dp Behring)					
Kaolin	0.04	0.63	0.64	0.69	0.6–1.4
Micronized kaolin	0.036	0.60	0.65	0.63	1.05*
Ellagic acid	0.086	0.60	0.67	0.70	1.07*
F XI:C (F XI dp Immuno)					
Kaolin	0.03	0.53	0.5	n.d.	—
Esterolytic F XI activity	0.17	0.51	0.57	0.52	0.76–1.1
F XI:Ag (RID)	0.89	0.89	0.86	0.98	0.6–1.36
Ratio F XI:C to F XI:Ag	0.04	0.71	0.74	0.7	0.87–1.11
F XII:C	0.32	0.85	1.4	0.69	0.56–1.96
F XII:Ag	0.4	0.87	1.4	0.58	0.45–2.14
Ratio F XII:C to F XII:Ag	0.8	1.0	1.0	1.2	—
HMWK:C	0.92	1.05	1.1	1.02	0.78–1.42
PK:C	1.3	1.4	1.42	1.4	0.85–1.95

Kaolin was used in a concentration of 20 mg/mL of saline. Micronized kaolin was adjusted to 5 mg/mL. Actin (Merz & Dade, Düringen, Switzerland) served as the source for ellagic acid.

*Value obtained with reference plasma 100% (Immuno AG, Vienna).

The first determinations of F XII:C were done only in Germany, using a one-stage F XII:C assay. According to these results, father, sister, and proposita appeared to have heterozygote factor XII deficiencies. During the follow-up study, the fresh blood samples obtained in 1985 were investigated in Austria by a two-stage F XII:C assay and F XII:Ag determinations. Interestingly, only the proposita's plasma was deficient in F XII:C and F XII:Ag with these assay systems on this occasion (Table 2).

Effect of Patient Plasma on Normal Plasma F XI Clotting Activity

No inhibitory activity of the proposita's plasma on normal plasma F XI could be discovered, as shown in Table 3.

Determination of F XI:Ag and F XII:Ag

All F XI-antigen determinations were carried out at least once with Bouma's antibody and once with the commercial antibody (Nordic) for comparison. Since no difference in the results was observed, all repeat experiments were performed with the easily accessible commercial product. F XI:Ag determined by RID was normal in all family members. The ratio of F XI:C to F XI:Ag was 0.04 in the proposita, 0.74 in

the mother, 0.7 in the father, and 0.71 in the sister. Comparison of F XI:C to F XI:Ag in 12 normal controls gave ratios between 0.87 and 1.11 (mean value 1.04 ± 0.15 , Table 2). Evaluation of F XI:Ag by neutralization-inhibition assay gave similar results, as published previously.¹⁹ F XII:Ag, measured by RID, was low in the patient, but normal in the other family members. The ratio of F XII:C to F XII:Ag was between 0.8 and 1.2 (Table 2).

Esterolytic Activity of F XI

Application of the chromogenic substrate method for measurement of factor XI activity gave good results when all sample dilutions were made with F XI-deficient plasma. Using this method of sample dilution, cleavage of S-2366 by any component other than factor XI was identical in all samples. Various mixtures of normal plasma and F XI-deficient plasma were evaluated (Table 4). Correct determinations of F XI above concentrations of 0.1 U/mL were possible with good correlation of the results with the clotting assay. However, if samples containing <0.1 U F XI/mL were assayed, eg, undiluted F XI-deficient plasma, determination of the esterolytic activity gave higher values than the clotting assay.

The values of the F XI:esterolytic activity in the patient's and her relatives' plasmas are shown in Table 2. According to these results, all family members were below the normal range.

Activation of F XI With Ellagic Acid and Micronized Kaolin

When micronized kaolin was used for the activation of F XI:C, the results were the same as with normal kaolin. However, with ellagic acid the estimated XI:C activity in the proposita was twice as high as with kaolin (Table 2). The reason for this phenomenon is presently unclear.

Effect of Kaolin on Normal and Patient's F XI

Incubation of normal plasma samples with kaolin resulted in fast (<5 minutes) removal of F XI antigen from the plasma supernatant. In contrast, the F XI antigen was still demonstrable in the patient's plasma supernatant (Fig 1). The father's F XI interacted normally with kaolin, whereas the mother's (Fig 1) and the sister's (Fig 2) F XI antigen was

Table 3. Effect of Patient Plasma on Normal Plasma F XI

Normal Plasma (%)	Patient Plasma (%)	Recovered F XI Activity (U/mL)
100	0	1.0
60	40	0.65
50	50	0.5
43	57	0.45
33	67	0.37
17	83	0.21
0	100	0.04

F XI activity was assayed after 1-hour incubation at 37°C in 1:5 diluted samples.

Table 4. Factor XI Esterolytic Activity and Clotting Activity in Mixtures of Normal Plasma and Factor XI Deficient Plasma

Sample		Theoretical Concentration of F XI in U/mL	F XI:Est Activity in U/mL	F XI:C Activity in U/mL
NP	XI dp			
—	+	0	0.067	0.01
1	7	0.125	0.14	—
1	3*	0.25	0.24	—
1	2	0.33	—	0.32
1	1	0.5	0.49	0.5
2	1	0.66	—	0.69
3	1	0.75	0.75	—
+	—	1.0	1.0	1.0

Standard curves were prepared with a standard reference plasma diluted with factor XI-deficient plasma.

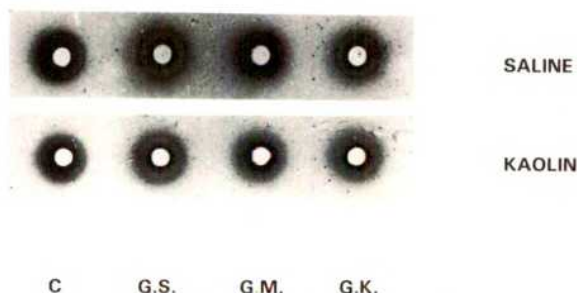


Fig 1. Radial immunodiffusion (RID) of kaolin-treated plasma supernatants. Undiluted plasma samples (100 μ L) were incubated with either 16 μ L of kaolin (20 mg/mL of saline) or 16 μ L of saline for 5 minutes at 37°C. Kaolin was separated by centrifugation for 2 minutes in a Beckman Microfuge B. Of each supernatant, 60 μ L was applied to 1% agarose plates containing 1.5% F XI antibody, and RID was carried out as described in the Materials and Methods Section. Samples, left to right: C, control plasma, G.S., proposita, G.M., father, G.K., mother.

not completely removed from the supernatant by kaolin. In HMWK-deficient plasma, studied as a control, the factor XI antigen did not interact with kaolin (Fig 2), as was expected.

Immunoblotting of Factor XI in Normal Plasma and Patient's Plasma

Samples containing 6 μ L of plasma were electrophoresed in a discontinuous buffer SDS-PAGE system and electrotransferred onto nitrocellulose. When they were reacted with the polyclonal F XI antibody followed by ^{125} I-factor XI, the autoradiograph showed essentially identical patterns in normal and patient's plasma (Fig 3). Four bands could be identified. One band, migrating with a mol wt of $\approx$ 130 kd, corresponded to purified factor XI. A second band, mol wt $\approx$ 160 kd, represented IgG. The other two bands could be identified in control experiments (data not shown) as HMWK, which ligated with ^{125} I-factor XI during the second incubation reaction.

Immunoblotting of Factor XI in Samples Passed Through DEAE-Sephadex A-50 Columns

Samples containing 60 μ L of the protein pool unbound by DEAE-Sephadex at pH 8.4 were used for SDS electrophoresis.

When normal plasma was chromatographed, these samples were pooled according to the F XI content determined in a clotting assay. When patient plasma was passed through the column, monitoring of the clotting activity was impossible due to starting activity that was too low. Therefore, the protein peak not bound to the column was pooled.

The samples were electrophoresed as described. The gels were electroblotted onto nitrocellulose and incubated with F XI antibody and ^{125} I-F XI. Figure 4 shows the results of the autoradiograph. In the normal material, a distinct band, corresponding to purified F XI, was present at mol wt $\approx$ 130 kd. A second diffuse band represented IgG. The two bands observed in plasma and identified as HMWK were no longer visible, which was not surprising since HMWK is known to bind to DEAE-Sephadex at pH 8.4. In the patient's plasma,

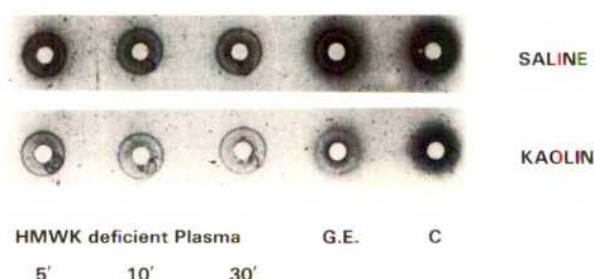


Fig 2. Radial immunodiffusion (RID) of kaolin-treated high-mol-wt kininogen (HMWK)-deficient plasma's supernatant. HMWK-deficient plasma (100 μ L) was treated with 16 μ L of kaolin (20 mg/mL of saline) or saline for 5, 10, and 30 minutes at 37°C. After centrifugation for 2 minutes in the Microfuge, 60 μ L of the supernatant was submitted to RID. Normal control plasma (C) and plasma of G.E. (proposita's sister) were treated identically before application to the RID plate.

only a very weak band migrating with mol wt $\approx$ 130 kd and corresponding to purified F XI could be distinguished. The band representing IgG was present in concentrations similar to those in the normal material. HMWK was also removed by DEAE-Sephadex at pH 8.4.

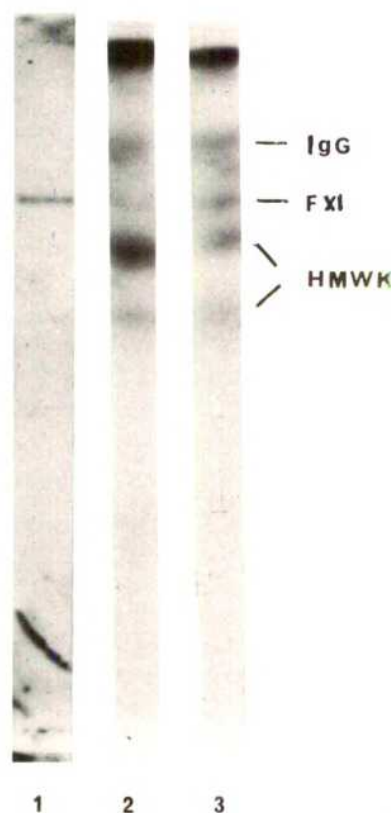


Fig 3. Immunoblotting of F XI in citrated plasma using 2% F XI antibody and ^{125} I-F XI overlay technique. Sixty-microliter aliquots of 1:10 diluted plasma were treated with 36 μ L of sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. Lane 3, G.S. plasma; lane 2, normal plasma, lane 1, purified F XI (2 μ g). Autoradiographs were exposed for 240 hours at -70°C .

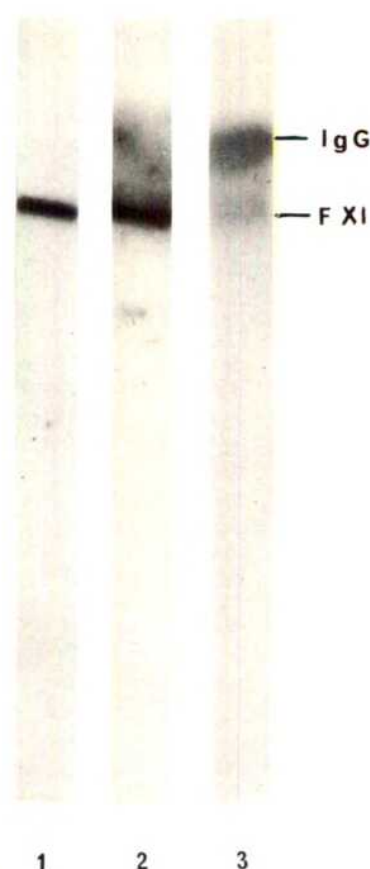


Fig 4. Immunoblotting of F XI in diethylaminoethanol (DEAE)-Sephadex-treated samples. Plasma samples were chromatographed on DEAE-Sephadex as described in the Materials and Methods section. Sixty-microliter aliquots of the unbound protein fraction of normal plasma (lane 2) and plasma of G. S. (lane 3) were treated with 36 μ L of 6% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using 2% F XI antibody and ^{125} I-F XI overlay technique. Lane 1 contained purified F XI (5 μ g). Autoradiographs were exposed for 110 hours at -70°C .

Immunoblotting of F XI in Kaolin Eluates

After incubation with kaolin, normal, the proposita's, and a F XI-deficient patient's plasma samples were centrifuged in a Microfuge. The kaolin pellets were washed and then eluted with SDS as described in the Materials and Methods section. Aliquots, containing 60 μ L of each kaolin eluate were subjected to SDS-PAGE and immunoblotting. Results are shown in Fig 5. F XI and/or F XIa, as well as cleaved HMWK, were present in the kaolin eluate from normal plasma. In the kaolin eluate of the proposita and the F XI-deficient patient, only cleaved HMWK could be observed.

DISCUSSION

Hereditary deficiencies of coagulation factors can be caused either by the absence of a clotting protein or by the synthesis of a defective molecule. For hereditary factor XI deficiency, present data agree that the protein is absent in these patients.^{8,9,11,15} Only one publication mentions two

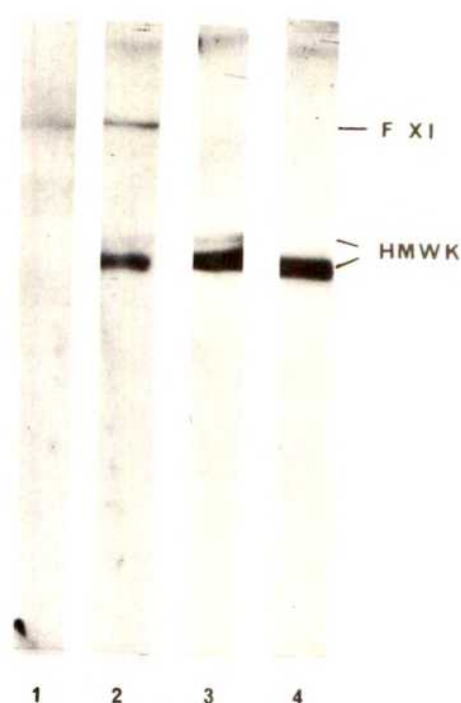


Fig 5. Immunoblotting of F XI in kaolin eluates using 2% F XI antibody and radiolabeled antigen (^{125} I-F XI) overlay technique. Lanes 2 through 4 contained 60 μ L of sodium dodecyl sulfate (SDS) eluates obtained from kaolin (320 μ g) adsorbed with 100 μ L of plasma (described in the Materials and Methods section). Lane 2, normal plasma; lane 3, G.S.; lane 4, factor XI-deficient patient. In lane 1, purified F XI (3 μ g) was run for comparison. Autoradiographs were exposed for 68 hours at -70°C .

patients, both of German origin, who apparently were positive for CRM.¹¹ Our report describes a German girl of non-Jewish ancestry with severe F XI:C deficiency but normal levels of F XI:Ag. Because a dysform of factor XI is a genetic rarity, our studies tried to confirm and elucidate the nature of the defect. Investigation of the effect of patient plasma on normal plasma ruled out an acquired or inherited inhibitor, which would have explained the phenomenon. General clotting tests and platelet function studies, carried out previously,¹⁹ showed that the only abnormalities were a mildly reduced F XII:C and a severely reduced factor XI:C. The factor XII:C level corresponded well with the factor XII:Ag, and therefore no further investigations of this protein were performed.

To shed some light on the patient's PTA defect, we studied the enzymatic properties of the factor XI protein in a chromogenic substrate assay. The study disclosed a fourfold higher esterolytic than clotting activity in the proposita. In contrast, in the other family members, the clotting activity was in the same range as the esterolytic activity. We believe that the observed elevation of F XI:est over F XI:C activity in the proposita's plasma is not an artifact, since we could accurately determine F XI:est activities >0.1 U/mL. The apparent F XI activity of 0.067 U/mL that we found in F XI-deficient plasma could not be inhibited by increased amounts of SBTI or CTI in the incubation mixtures, and its source is presently unknown.

The interesting phenomenon of a higher esterolytic than clotting activity in the plasma of G.S. may have various causes. First, the proband's molecule could be structurally defective and therefore no longer able to activate its natural substrate, factor IX, while it still cleaves the chromogenic substrate. Our results do not rule out this possibility.

Second, the reduced F XII level in the patient's plasma may have affected the clotting assay more than the esterolytic test, but did not. Reconstitution of the patient's plasma with purified F XII to normal concentration did not result in an increase of F XI:C activity.

Third, the defect in the proband's F XI molecule may have made an interaction with either HMWK and/or the negatively charged surface (kaolin) impossible. As a consequence, factor XI would remain in solution, and its activation by factor XIIa would occur at a slower rate. The six times longer activation time in the esterolytic test (30 minutes v 5 minutes in the clotting assay) could allow an increased generation of F XIa. Unfortunately, the incubation time of the clotting assay cannot be prolonged due to the presence of all natural inhibitors. Inactivation of the inhibitors by pretreatment of the plasma with CHCl_3 as used in the esterolytic test, is not applicable in the clotting assay. Thus, an approximation of the incubation time of both assay systems cannot be achieved.

Our observation that F XI antigen was not adsorbed to kaolin from G.S.'s plasma indicated that some binding properties of the F XI molecule were defective. Because binding of F XI to HMWK is required for the adsorption of F XI from plasma to negatively charged surfaces,^{20,23} the defect appeared to be due to an impaired F XI-HMWK complex formation.

To appreciate the interaction of the patient's F XI with HMWK and surface, performance of direct binding experiments with purified proteins would be important. Presently, however, we do not have enough plasma for purification of this abnormal protein.

A direct examination of the material adsorbed onto the kaolin surface was accomplished by SDS-PAGE and immunoblotting of kaolin eluates (Fig 5). In normal plasma, we could demonstrate adsorption of F XI and HMWK onto kaolin. HMWK was found in the kaolin eluate in its cleaved form and migrated as two close bands with a mol wt of ≈ 95 kd in unreduced samples. F XI and/or F XIa was identified as one band with an apparent mol wt of ≈ 150 kd in the unreduced eluates.

In the kaolin eluates of the *proposita*, only HMWK was present, and it exhibited the same electrophoretic behavior as in normal plasma. This observation confirmed our previous results obtained by RID which showed that F XI-antigen was not removed from plasma of G.S. by kaolin. The immunoblotting data that prove normal kaolin binding of HMWK support our hypothesis that the F XI molecule is unable to bind to HMWK and thus is not adsorbed to the kaolin surface. In the kaolin eluate of the truly F XI-deficient patient's plasma, only HMWK was demonstrable, as was expected.

The critical proof of the presence of an inactive F XI in the plasma of the *proposita* was approached not only by RID, but

also by immunoblotting studies. Immunoblotting studies of complex protein mixtures, eg, plasma, are difficult, especially if a trace protein such as F XI is detected. Therefore, in the case of F XI, the polyclonal antibody was preadsorbed with F XI-deficient plasma, previously immobilized onto nitrocellulose membranes, to remove contaminating antibodies not directed against F XI. Because this pretreatment alone was not sufficient to eliminate all nonspecific bands, a radioactive overlay technique using ^{125}I -FXI was applied as described by Lämmle and colleagues.²⁴ This technique improved the specificity of the method and allowed detection of F XI in a volume of 6 μL of plasma. Similar concentrations of F XI with the same electrophoretic migration behavior were present in the plasma of the *proposita* and in a normal control plasma (Fig 3). In both plasmas, the incubation of the blot with ^{125}I -F XI resulted in the appearance of two bands representing HMWK. The reason for this phenomenon is the complex formation between HMWK and F XI, a well-established reaction.²⁰ The observation of a normal complex formation between the patient's HMWK and purified ^{125}I -F XI clearly shows that the patient's HMWK has normal F XI binding properties. Therefore, the binding defect must be located in the F XI molecule.

Interestingly, in both plasma samples, a band corresponding to human IgG was visible, even when the ^{125}I -F XI overlay technique was used. The IgG band and the two HMWK bands were also seen in a control experiment in which a plasma blot, not treated with antibody, was incubated with ^{125}I -F XI. Thus, it appears that F XI not only complexes with HMWK but also attaches to IgG. Further experiments to study this phenomenon are necessary.

When normal and patient plasma were chromatographed on DEAE-Sephadex at pH 8.4 and the unadsorbed protein fractions were analyzed on SDS-PAGE followed by immunoblotting, we made an interesting observation. In normal plasma, F XI as well as IgG did not bind to DEAE-Sephadex at pH 8.4, and both bands could be identified on the autoradiograph (Fig 4). In contrast, in the plasma of the *proposita*, IgG passed through the column unadsorbed, whereas F XI apparently was removed by DEAE-Sephadex. We cannot yet show data that prove the binding of F XI to DEAE-Sephadex, however. Experiments to confirm these results are in progress.

An attempt to demonstrate F XI in the plasma of G.S. by Laurell immunoelectrophoresis was not successful (data not shown); no rockets could be obtained. The inability of the F XI of the *proposita* to form a complex with HMWK explains these results. F XI does not migrate under the conditions used in the Laurell techniques unless it is bound to HMWK.²⁵

The defective surface adsorption properties in the proband's factor XI molecule are a very interesting phenomenon, which may lead to better insights into the factor XI-HMWK-surface interactions.

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Anemia of the Belgrade Rat: Evidence for Defective Membrane Transport of Iron

By Barbara J. Bowen and Evan H. Morgan

The mechanisms underlying the impaired utilization of transferrin-bound iron by erythroid cells in the anemia of the Belgrade laboratory rat were investigated using reticulocytes from homozygous anemic animals and transferrin labeled with ^{59}Fe and ^{125}I . The results were compared with those obtained using reticulocytes from phenylhydrazine-treated rats and iron-deficient rats. Each step in the iron uptake mechanism was investigated, ie, transferrin-receptor interaction, transferrin endocytosis, iron release from transferrin, and transferrin exocytosis. Although there were quantitative differences, no fundamental difference was found in any of the abovementioned aspects of cellular function when the reticulocytes from Belgrade rats were

compared with those from iron-deficient animals. The basic defect in the Belgrade reticulocytes must therefore reside in subsequent steps in iron uptake, after it is released from transferrin within endocytotic vesicles, ie, in the mechanism by which it is transferred across the lining membrane of the vesicles into the cell cytosol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of reticulocyte ghosts extracts demonstrated a prominent protein band of mol wt 69,000 that was absent or present only in low concentration extracts from the other two types of reticulocytes. This may be a result of the genetic defect.

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THE BELGRADE laboratory rat suffers from hereditary microcytic, hypochromic anemia inherited as an autosomal recessive trait. The anemia is accompanied by reticulocytosis, elevated plasma iron and iron-binding capacity, decreased stainable iron in the tissues, and decreased growth rate.^{1,2} Iron uptake from transferrin by erythropoietic cells is diminished and globin synthesis is defective.^{1,3-5} The mechanisms underlying the impaired utilization of iron are unknown.

From results obtained in previous investigations using reticulocytes and transferrin labeled with ^{125}I and ^{59}Fe , it was concluded that uptake of the transferrin-iron-receptor complex is unimpaired and that the abnormality of iron incorporation probably resides in defective release of iron from transferrin within endocytotic vesicles. However, examination of the transferrin uptake curves presented in this article suggests a decreased rate of uptake by Belgrade reticulocytes when compared with those from control animals. If this is true, abnormal transferrin-receptor interaction or impaired endocytosis of the transferrin-receptor complex may be responsible. No investigations of the affinity of the receptors for transferrin have been published. Endocytosis of transferrin by Belgrade reticulocytes was studied by electron microscopy using antitransferrin labeled with ferritin.⁶ However, this investigation did not allow the rate of endocytosis to be measured. Moreover, even if transferrin-receptor interaction and the rate of endocytosis are normal, the defect in iron uptake could be due to defective transport of iron across the

endocytotic vesicle membrane instead of an abnormality of iron release from transferrin, as has been proposed.⁵

The aim of the present investigation was to resolve the above questions by studying each stage in the process of iron uptake by reticulocytes, ie, transferrin-receptor interaction, endocytosis, iron release from transferrin, membrane transport of iron, and exocytosis of transferrin.⁷ Early in this work, it became apparent that the appropriate control for studies with Belgrade reticulocytes is iron-deficient reticulocytes rather than those from iron-replete animals. Hence, the investigation was extended to include studies with reticulocytes from iron-deficient rats. The results indicate that the basic defect in the Belgrade reticulocyte lies in the mechanism of iron transfer across the lining membrane of endocytotic vesicles.

MATERIALS AND METHODS

Animals. Heterozygous (b/-) male and female Belgrade rats were very kindly provided by Dr John A Edwards, Department of Medicine, State University of New York at Buffalo. These animals were used to produce a colony of anemic (b/b) and nonanemic (b/-, +/-) animals. During the first 3 months of life and during pregnancy, the anemic animals were given frequent intraperitoneal (IP) injections of fresh blood from nonanemic rats of the same strain to reduce the high mortality that results from the severe anemia. The animals were fed a standard laboratory rat diet.

Iron deficiency was produced in 3-month-old male nonanemic Belgrade rats by the removal of 5 mL of blood by heart puncture on four occasions at 1-week intervals while maintaining the rats on an iron-deficient diet.⁸ The animals were then kept on this diet for a further 2 months, being bled only as required for the studies described below.

Reticulocytosis was also induced in nonanemic (b/- and +/-) rats by a single IP injection of neutralized phenylhydrazine HCl (10 mg/100 g of body weight). The animals were bled by heart puncture (2 mL/100 g of body weight) 5 days later and again 2 days after the first bleed. This second sample of blood was used for the experiments described in this article. In one experiment, reticulocytosis was induced solely by bleeding by heart puncture (2 mL/100 g of body weight) three times in 1 week of (+/-) rats that had been injected intramuscularly (IM) with 50 mg iron as iron-dextran 1 week before the first bleed. Blood was obtained 3 days after the last bleeding for the iron uptake studies.

For brevity, the reticulocyte-rich blood cells from homozygous Belgrade (b/b) rats, heterozygous (b/-) rats, iron-deficient (+/-)

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rats, and phenylhydrazine-treated (?/–) rats are referred to as Belgrade, heterozygous, iron-deficient, and control reticulocytes, respectively.

Purifications and labeling of transferrin. Rat and rabbit transferrins were isolated from plasma by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose (DE52, Whatman) and gel filtration with Ultrogel ACA34 (LKB).⁹ The rat transferrin was labeled with ⁵⁹Fe and ¹²⁵I as described previously.⁹ It was used in the diferric form in the experiments described below. The rabbit transferrin was used in cold-chase experiments and in the preparation of antisera in goats by three intramuscular injections of 5-mg quantities of the protein in Freund's complete adjuvant at 2-week intervals.

Isolation of transferrin receptor. The transferrin receptor was isolated from the placentas of control rats on the nineteenth day of gestation. The placentas from four pregnant rats were washed with several changes of phosphate-buffered saline (PBS) and then homogenized with 3 vol of PBS using a Potter-Elvehjem homogenizer. The insoluble components of the placentas were sedimented by centrifugation for 30 minutes at 40,000 g (average) and washed three times by resuspension in PBS and centrifugation as before. They were then treated with 2% Triton X-100 (Sigma, St Louis), 1 mmol/L of iodoacetic acid, 1.0 mmol/L of phenylmethylsulfonyl fluoride in PBS for 1 hour, followed by centrifugation at 40,000 g (average) for 1 hour. The transferrin receptor was isolated from the supernatant solution by affinity chromatography using rat transferrin coupled to Sepharose 4B (Pharmacia, Piscataway, NJ) following the procedure described by van Driel and colleagues.¹⁰ The isolated receptor was contaminated with rat transferrin, which was largely removed by passage through a Sepharose 4B-anti-rat transferrin column equilibrated and washed with PBS, pH 7.4-0.2% Triton X-100. All procedures were performed at 4°C.

Measurement of transferrin and iron uptake. Blood was obtained from the anemic rats by heart puncture, using heparin as an anticoagulant. The cells were washed three times with ice-cold PBS and were then suspended in Hanks' and Wallace balanced salt solution. The buffy coat was removed from the RBC layer at the end of each wash. After the third wash, the cells were centrifuged at 2,000 g for 30 minutes at 4°C and the top one-quarter of the RBC layer was removed to obtain a reticulocyte-enriched cell suspension. These cells were suspended in Hanks' solution. The reticulocyte counts of these suspensions varied from 20% to 38% for the Belgrade reticulocytes, 21% to 49% for heterozygous reticulocytes, 22% to 59% for control reticulocytes and 18% to 37% for iron-deficient reticulocytes.

Aliquots of the reticulocyte suspensions were then incubated with ¹²⁵I-⁵⁹Fe-labeled transferrin, washed with ice-cold PBS and treated with Pronase (Calbiochem-Boehringer, San Diego) at 4°C to measure endocytosed transferrin as previously described.¹¹ Transferrin and iron exocytosis were measured by a cold-chase procedure, again using incubation with Pronase at 4°C to distinguish between membrane-bound and intracellular transferrin.¹¹ The validity of this method for the measurement of transferrin endocytosis has been demonstrated by quantitative electron microscope autoradiography.¹² To measure the number and affinity of transferrin receptors on the cell surface, samples of the cells were incubated for 30 minutes at 4°C with varying concentrations of radiolabeled transferrin in the presence of 10 mg/mL of bovine serum albumin (BSA) (Sigma, St Louis, Type A7030). Total cell-associated transferrin was then determined by centrifuging 150-μL aliquots of the cell suspension through a mixture of di-n-butyl phthalate and dinonyl phthalate (2:1; vol:vol) in a Beckman Microfuge for 15 seconds, and counting the resultant cell pellet for radioactivity. Nonspecific binding of transferrin was measured in duplicate incubations con-

taining a 50-fold molar excess of unlabeled diferric rat transferrin. Specific binding was calculated as the difference between total and nonspecific binding, and was used to estimate the numbers of specific receptors on the cell membrane (B_{max}) and the association constant of the transferrin-receptor reaction (K_a) by Scatchard method.¹³ The total number of cellular receptors was measured in a similar manner except that the incubations were performed at 37°C. The percentage of total receptors situated on the cell membrane was calculated from these values.

Analytical procedures. Reticulocytes were determined by staining with new methylene blue, packed cell volume by the microhematocrit procedure and mean corpuscular volume (MCV) by the use of an automated cell counter (Model H.1; Technicon, Terrytown, NY). Heme was extracted from washed cells by the method of Thunell.¹⁴ Radioactivity was counted in a three-channel γ-scintillation counter. Polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli,¹⁵ using 10% polyacrylamide in the resolving gel, and reducing the samples by heating to 95°C in sample buffer containing 4% sodium dodecyl sulfate (SDS) and 10% mercaptoethanol. The samples were run in duplicate. One set of the duplicates was stained for protein using Coomassie brilliant blue R250. The transferrin receptor was identified in the other set of the duplicates by Western blot transfer of the proteins from the polyacrylamide gel to nitrocellulose membrane and by staining with anti-rat transferrin receptor serum, followed by reaction with goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad, Richmond, CA). The anti-rat transferrin receptor serum was produced in rabbits by injection of three doses of 100 μg of the receptor in Freund's complete adjuvant. The antiserum was cleared of contaminating anti-rat transferrin by passage through a column of transferrin coupled to Sepharose 4B.

RESULTS

Transferrin receptors. The affinities and numbers of transferrin receptors on Belgrade, iron-deficient, and control reticulocytes are given in Table 1. There were no significant differences ($P > .05$) between the K_a values for any of the three types of cells. Although the mean number of total sites per cell was significantly higher for the control reticulocytes than for the other two types ($P < .001$), the numbers on the outer cell membrane showed no significant differences ($P > .05$). Belgrade and iron-deficient reticulocytes had higher percentages of their total receptors on the cell membrane (57% and 69%), respectively, than did the control reticulocytes (30%). This difference was confirmed in experiments in which measurements were made before and after

Table 1. Transferrin Receptor Affinities (K_a) and Numbers on Outer Cell Membrane, and Total Receptor Numbers for Belgrade, Iron-Deficient, and Control Reticulocytes

Reticulocytes	Transferrin Receptors		
	K_a (M^{-1}) ($\times 10^{-6}$)	Numbers (Molecules/Cell $\times 10^{-4}$)	
		Outer Cell Membrane	Total
Belgrade	8.10 $\pm$ 1.25(3)	2.60 $\pm$ 0.23(7)	3.81 $\pm$ 0.42(7)*
Iron-deficient	7.13 $\pm$ 0.80(3)	2.86 $\pm$ 0.33(5)	5.00 $\pm$ 0.46(5)*
Control	7.87 $\pm$ 1.32(3)	2.85 $\pm$ 0.28(7)	9.66 $\pm$ 0.61(7)

Each value is the mean ($\pm$ SEM) of the number of measurements shown in parentheses. The K_a values and outer membrane receptor numbers were obtained by incubation of cells with ¹²⁵I-labeled transferrin at 4°C, and the total receptor numbers were obtained by incubation at 37°C (described in text).

*Significant difference from control reticulocytes, $P < .01$.

incubating reticulocytes with Pronase at 4°C to remove surface receptors. This produced only a 22% reduction in total receptor numbers on the control reticulocytes but 55% and 71% reductions for the iron-deficient and Belgrade reticulocytes, respectively (means of two experiments). The total number of receptors in the three types of reticulocytes correlated to some extent with the mean MCV values, which were 21, 27, and 85 fL for the Belgrade, iron-deficient, and control reticulocytes, respectively. The MCV of mature erythrocytes from untreated nonanemic rats was 59 fL.

In the one experiment in which reticulocytes from the rats that had been bled >1 week after receiving an iron-dextran injection were used, the K_a and receptor number values and the rates of iron and transferrin uptake were similar to those from the control reticulocytes.

The transferrin receptors were also examined by SDS-PAGE (Fig 1). The receptors from all cell types, including known heterozygous (B/−) rats), were identified as two closely opposed bands of similar mol wt, ~92,000. Although not evident in Fig 1, in some electrophoretic runs the Belgrade (B/B) receptor bands were of slightly faster mobility than the receptor from control reticulocytes. However, in these cases the receptor from the iron-deficient reticulocytes had mobility identical to that from the Belgrade cells. Hence, the change observed with the Belgrade reticulocytes appeared to be a consequence of iron-deficient erythropoiesis rather than of a genetic defect.

Several other differences were noted between the electrophoretic patterns of extracts from the ghosts of Belgrade and control reticulocytes. However, the same differences, with one exception, were noted when extracts of iron-deficient ghosts were compared with controls. The exception was the appearance of a strong protein band of approximate mol wt 69,000 in the Belgrade extracts as compared with its absence or near absence in the extracts from the other two types of

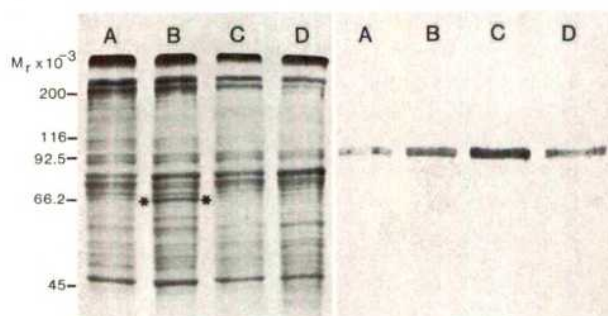


Fig 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunochemical staining of the transferrin receptors after Western blot transfer to a nitrocellulose membrane of Triton X-100 extracts of the ghosts of iron-deficient (A), Belgrade (B), control (C), and heterozygous (D) reticulocytes. The cells were hemolysed with 15 mM phosphate buffer and washed three times in the same buffer; the ghosts were then extracted with 2% (wt:vol) Triton X-100 in the buffer. The Triton X-100 extract was centrifuged at 40,000 g for 1 hour at 4°C, and the supernatant was used for electrophoresis. Asterisks indicate the protein present in high concentration only in extracts of Belgrade reticulocytes. The transferrin receptor is the double band at the 92.5-mol wt position as shown by Western blot.

reticulocytes including those from heterozygous (+/−) Belgrade rats (Fig 1).

Transferrin endocytosis and iron uptake. The rates of transferrin endocytosis and iron uptake were measured in eight separate experiments comparable to the one shown in Fig 2. As noted by earlier investigators, the outstanding feature was the relatively slow rate of iron uptake by Belgrade reticulocytes, even when compared with iron-deficient animals. Transferrin endocytosis was also slower in the Belgrade reticulocytes, but the relative decrease was not as great as that of iron uptake. This is illustrated very clearly by comparison of the mean rates of iron uptake and transferrin endocytosis in these experiments (Table 2). The mean rates of iron uptake obtained for the control and iron-deficient reticulocytes were almost twice those of transferrin endocytosis (Table 2), indicating that each molecule of diferric transferrin donated both its iron atoms to the cells during each intracellular cycle, as has been reported previously for rabbit reticulocytes.¹¹ However, with Belgrade reticulocytes the mean rate of iron uptake was only 0.7 times that of transferrin. Hence, on the average, only ~35% of the iron carried into the cells by endocytosis of transferrin was being retained by the cells. Incorporation into heme of the iron taken up by the cells was also measured. The proportion of iron incorporated into heme during 30-minute incubation at 37°C was much lower for the Belgrade and Fe-deficient cells (9% to 30%) than for the controls (39% to 81%).

Two other experiments using agents known to affect the rates of transferrin endocytosis and iron uptake, phorbol 12-myristate 13-acetate (PMA)¹⁶ and hemin,¹⁷ were also performed. PMA produced increases in the rates of transferrin endocytosis by all three types of cells (Table 3). In control and iron-deficient reticulocytes, this was accompanied by an increase in the rate of iron uptake, but with Belgrade reticulocytes no such increase was observed. In contrast, hemin inhibited transferrin endocytosis, and the degree of inhibition was greater with the control reticulocytes than with the other two types. In iron-deficient cells, the rate of iron uptake was inhibited to almost the same

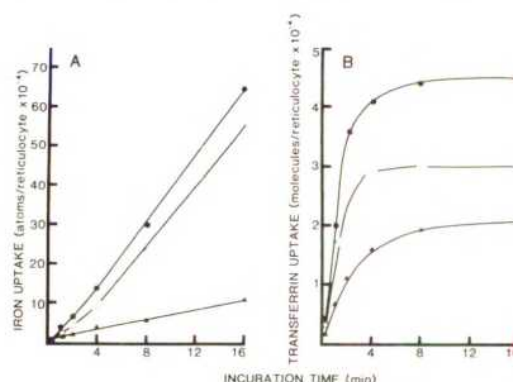


Fig 2. Iron (A) and transferrin (B) uptake by control (●), iron-deficient (○), and Belgrade reticulocytes (▲). Internalization of iron and transferrin during incubation at 37°C with 2.5 μ mol/L ^{59}Fe - ^{125}I transferrin is shown. The reticulocyte counts were 25% for the control reticulocytes and 28% for the other two types of cells.

Table 2. Rates of Iron Uptake and Transferrin Endocytosis and the Ratio of Two Measurements for Belgrade, Iron-Deficient, and Control Reticulocytes

Reticulocytes	Iron Uptake (Atoms/Cell/ min $\times 10^{-4}$)	Transferrin Endocytosis (Molecules/Cell/ min $\times 10^{-4}$)	Iron:Transferrin
Belgrade	$0.68 \pm 0.082^{*}\dagger$	$1.05 \pm 0.21\dagger$	$0.65 \pm 0.13^{*}\dagger$
Iron-deficient	$2.33 \pm 0.69\dagger$	1.18 ± 0.31	1.97 ± 0.56
Control	4.05 ± 0.47	2.10 ± 0.25	1.93 ± 0.35

Each value is the mean ($\pm$ SEM) of eight measurements with Belgrade and control reticulocytes and five measurements with iron-deficient reticulocytes.

*Significant difference from control reticulocytes, $P < .01$.

$\dagger$ Significant difference from iron-deficient reticulocytes, $P < .01$.

$\ddagger$ Significant difference from control reticulocytes, $P < .05$.

degree as that of transferrin endocytosis. However, with Belgrade reticulocytes there was little change in the rate of iron uptake. Hence, the rate of iron uptake relative to that of transferrin endocytosis increased considerably (Table 3).

The rates of transferrin endocytosis and iron uptake were also measured in two experiments in which heterozygous reticulocytes were compared with Belgrade and control cells. The results confirmed the abovementioned differences between Belgrade and control reticulocytes but showed no differences between control and heterozygous reticulocytes.

Transferrin exocytosis and iron release from reticulocytes. The rates of transferrin exocytosis and iron release from reticulocytes labeled with ^{59}Fe and ^{125}I -transferrin by prior incubation with doubly labeled transferrin were measured in four experiments similar to that shown in Fig 3. In all cases, the rate of transferrin exocytosis was as fast from the Belgrade and iron-deficient reticulocytes as from the control cells. However, in confirmation of earlier observations,^{4,6} the Belgrade reticulocytes released relatively more of their radioiron than did the control reticulocytes. This was not a simple consequence of iron deficiency since it was not observed with the iron-deficient cells.

Table 3. Effects of PMA and Hemin on Iron Uptake and Transferrin Endocytosis by Belgrade, Iron-Deficient, and Control Reticulocytes

Reticulocytes	Treatment	Iron Uptake (Atoms/Cell/ min $\times 10^{-4}$)	Transferrin Endocytosis (Molecules/Cell/ min $\times 10^{-4}$)	Iron: Transferrin
Belgrade	Control	0.60	1.05	0.57
	PMA	0.60	1.25	0.48
Iron-deficient	Control	2.12	1.14	1.86
	PMA	2.50	1.38	1.81
Control	Control	4.87	2.45	1.99
	PMA	5.69	2.97	1.92
Belgrade	Control	0.85	0.78	1.07
	Hemin	0.77	0.42	1.81
Iron-deficient	Control	2.40	1.14	2.10
	Hemin	1.16	0.63	1.84
Control	Control	4.86	2.53	1.92
	Hemin	1.25	0.66	1.88

Reticulocytes were preincubated with PMA (10^{-8} mol/L) or hemin (4×10^{-5} mol/L) for 15 minutes at 37°C prior to the addition of labeled transferrin. Results are from two separate experiments.

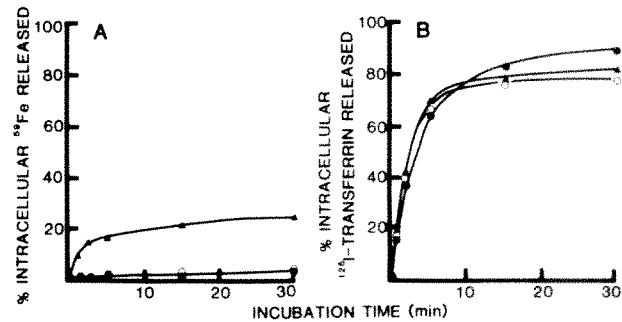


Fig 3. Release of intracellular ^{59}Fe and ^{125}I -transferrin (B) from control (●), iron-deficient (○), and Belgrade (▲) reticulocytes. The cells were labeled by incubation with $5 \mu\text{mol/L}$ of diferric ^{59}Fe - ^{125}I -transferrin for 20 minutes at 37°C . After being washed, they were reincubated in the presence of 10 mol/L of unlabeled diferric transferrin, and the loss of intracellular ^{59}Fe and ^{125}I -transferrin was measured by the Pronase method. Results are expressed as percentage of label at the commencement of reincubation. The reticulocyte counts were 45%, 35%, and 26% for the control, iron-deficient, and Belgrade cells, respectively.

Iron release from transferrin. The above experiments showed that the rate of iron accumulation by Belgrade reticulocytes is less than one-half that which would be expected from the rate of endocytosis of transferrin and that a significant proportion of the iron taken up by these cells is later returned to the extracellular medium. These results suggested that the Belgrade reticulocytes were defective in releasing iron from transferrin within the endocytotic vesicles or that the iron after its release was unable to pass through the lining membranes of the vesicles into the cell cytosol. Two types of experiments were used to distinguish these possibilities. The first was based on the observation that certain ferrous iron chelators such as 2,2'-bipyridine are able to chelate iron after it has been released from transferrin by the action of reticulocytes and to transport it from the cells, thus preventing its accumulation by the cells.¹⁸ The chelator-bound and transferrin-bound iron can be distinguished by treatment with ice-cold ethanol, which precipitates transferrin-iron quantitatively but does not precipitate bipyridine-iron.¹⁹ The second approach was to perform cold-chase experiments with rabbit rather than rat transferrin so that radioiron that has been released from ^{59}Fe -labeled rat transferrin and bound by the rabbit protein can be distinguished by precipitation of the rabbit protein with a specific anti-rabbit transferrin antiserum. A similar procedure has been used for other purposes with human and rabbit transferrin and rabbit reticulocytes.²⁰

When control and iron-deficient reticulocytes were incubated with ^{59}Fe - ^{125}I -rat transferrin in the presence of 2,2'-bipyridine, the radioactive iron was transferred to bipyridine at almost the same rate as it was accumulated by reticulocytes in the absence of bipyridine (Fig 4). However, with Belgrade reticulocytes, the rate of transfer of ^{59}Fe to bipyridine was 2.7 ± 0.25 times as fast as iron accumulation by control cells (mean $\pm$ SEM of eight measurements). No transfer to bipyridine occurred in the absence of cells, and with all cell types the transfer was inhibited by NH_4Cl (15

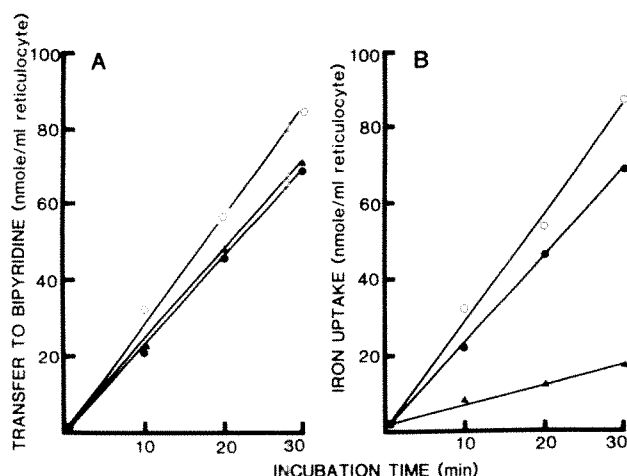


Fig 4. Transfer of iron 2,2'-bipyridine (A) and iron uptake (B) by control (●), iron-deficient (○), and Belgrade reticulocytes (▲). The cells were incubated with 5 $\mu\text{mol/L}$ of diferric ^{59}Fe - ^{125}I -transferrin in the presence (A) or absence (B) of 1 mmol/L of 2,2'-bipyridine. Iron transfer to bipyridine was measured by precipitation of transferrin-bound ^{59}Fe in the incubation solution with ethanol, and iron uptake by the cells was measured after washing as described in the text. The reticulocyte counts were 36%, 26%, and 25% for the control, iron-deficient, and Belgrade cells, respectively.

mmol/L), nigericin (10 $\mu\text{mol/L}$), monensin (10 $\mu\text{mol/L}$) and *N*-ethylmaleimide (2 mmol/L), which also inhibited iron uptake by cells incubated without bipyridine.

The results of one cold-chase experiment with rabbit transferrin are shown in Fig 5. Similar results were obtained in a second such experiment. The fraction of intracellular ^{59}Fe released from the cells during 5-minute cold chase with rabbit transferrin varied with the duration of the period used to label the cells with radiolabeled transferrin. It was low with both control and iron-deficient reticulocytes but high with Belgrade reticulocytes. The values obtained with the first two types of cells changed from 8% to 1% as the uptake time was increased from 1 to 16 minutes. However, with Belgrade reticulocytes, 50% to 54% of the radioiron taken up during 1 to 4 minutes was released and 24% of that was taken up during 16 minutes. Of this ^{59}Fe , 74% to 77% was present on the chase rabbit transferrin, indicating that it had been released from its initial complex with rat transferrin during the intracellular cycle. A control experiment was performed by incubating 1, 2, 5 and 10 μg ^{59}Fe - ^{125}I -labeled rat transferrin with 1 mL of 2.5 mol/L of 50% iron-saturated rabbit transferrin for 10 minutes at 37°C followed by precipitation of the rabbit transferrin with specific antiserum under the conditions used in the above experiments. Only 0.8% to 1.6% of the ^{59}Fe was precipitated by the antiserum. Hence, the results of these experiments confirm the conclusions from the bipyridine experiments that Belgrade reticulocytes are unimpaired in their ability to release iron from transferrin.

DISCUSSION

The results of these experiments demonstrate that the basic defect of iron metabolism inherent in the Belgrade reticulocytes is an impairment of iron transfer into the cells

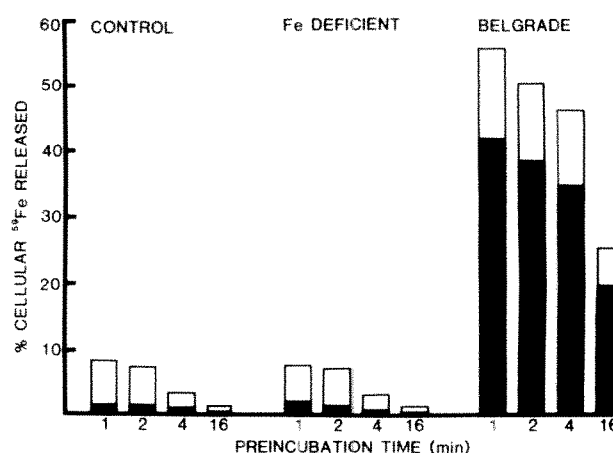


Fig 5. Effect of time of preincubation with diferric ^{59}Fe - ^{125}I -transferrin on the release of intracellular ^{59}Fe from control, iron-deficient, and Belgrade reticulocytes. The cells were incubated for the indicated times at 37°C with 2.5 $\mu\text{mol/L}$ of ^{59}Fe - ^{125}I -transferrin, washed, treated with 1% Pronase at 4°C to remove surface-bound transferrin, rewashed, and then reincubated with 2.5 $\mu\text{mol/L}$ of 50% iron-saturated nonradioactive rabbit transferrin for 10 minutes at 37°C. Cells and supernatant were separated by centrifugation, 1.5 mg nonradioactive diferric transferrin was added, and the rabbit transferrin was precipitated from the supernatant with goat anti-rabbit transferrin serum. The total height of each column indicates the amount of ^{59}Fe released from the cells and the dark portion of the columns indicates the amount precipitated with the anti-rabbit transferrin serum. Less than 3% of the ^{125}I -rat transferrin released from the cells was precipitated by the antiserum.

after it has been released from transferrin. Iron release from transferrin occurs within endocytotic vesicles.^{7,21} Hence, the site of impaired transfer can be concluded to be the lining membrane of these vesicles. The membrane may contain a carrier for iron that is defective as a consequence of the genetic abnormality expressed in homozygous (b/b) Belgrade rats. Because evidence shows that the iron of transferrin is reduced to the ferrous state within the endocytotic vesicles²² the form of iron transported by the carrier may be Fe^{II} .

Unimpaired endocytosis of transferrin associated with diminished iron uptake and increased iron release from Belgrade cells indicates that the abnormality lies in the metabolism of transferrin-iron after its endocytosis. Although the mechanism of action of bipyridine on iron uptake by reticulocytes is uncertain, considerable evidence shows that it acts after iron has been released from transferrin by the cells.^{18,19} Hence, the results from the experiments with 2,2'-bipyridine and the cold chase with rabbit transferrin clearly show that the amount of iron released from transferrin during its endocytotic cycle in Belgrade reticulocytes is far in excess of that which becomes fixed by the cells. Indeed, the amount released is equivalent to that which can be accounted for by the measured rate of transferrin endocytosis. Also, this release is inhibited by the lysosomotropic agent NH_4Cl and the proton ionophores nigericin and monensin, indicating that the release process is as dependent on acidification of the endocytotic vesicles as it is in normal reticulocytes.²¹ Hence, there is no evidence for a

decreased capacity of Belgrade reticulocytes to release iron from transferrin.

The results of the experiments with PMA and hemin are compatible with the above conclusions and explain why hemin has little effect on iron uptake by Belgrade reticulocytes.²³ As previously reported,¹⁶ PMA stimulated transferrin endocytosis and iron uptake by control and iron-deficient reticulocytes but had no effect on iron uptake by Belgrade reticulocytes even though the rate of endocytosis was accelerated in these cells. Presumably, the defect in membrane transport of iron prevented the cellular uptake of the increased amount of iron being endocytosed with the transferrin. In contrast, hemin inhibits the rate of transferrin endocytosis and recycling.¹⁷ Because these processes are the rate-limiting steps in iron uptake by normal and iron-deficient reticulocytes, there is a proportionate reduction in iron uptake. However, with the Belgrade reticulocytes, the rate-limiting step is the membrane transfer of iron. Therefore, reduced rate of delivery of iron to the site of this transfer by endocytosis would have little effect on the rate of iron uptake until the delivery became rate limiting.

This investigation confirms and extends an earlier report that iron-deficient rat reticulocytes show several alterations in iron metabolism when compared with iron-replete reticulocytes,²⁴ and demonstrates that similar changes occur whether the iron-deficient cells are the result of a genetic defect or are due to iron depletion produced by dietary means combined with bleeding. Thus, total transferrin receptor numbers were lower, but a greater proportion of the receptors were on the outer cell membrane in iron-deficient than in normal reticulocytes. This was accompanied by a diminished rate of iron uptake and impaired incorporation of iron into heme.

Whether the control reticulocytes used in the present work were normal cells is debatable. Because they were collected 7 days after the injection of phenylhydrazine, it is unlikely that they were directly affected by the drug. This is supported by

the observations that their transferrin receptor numbers, distribution, and function were similar to those of iron-replete reticulocytes produced solely by bleeding. However, the control reticulocytes were undoubtedly produced as a consequence of greater erythropoietic stimulation than that received by the iron-deficient and Belgrade cells and hence were probably more immature than the latter cells. This would account for their relatively large size when compared with mature rat erythrocytes and for some of the quantitative differences between the control and other types of reticulocytes. However, the changes in the efficiency of iron incorporation into heme, transferrin receptor distribution, and electrophoretic appearance of cell membrane extracts in the Belgrade and iron-deficient reticulocytes when compared with the control cells are likely to be a consequence of iron deficiency rather than of cell maturity. Such changes were not observed in any samples of control reticulocytes even though reticulocyte count and, presumably, cell maturity varied considerably from one sample to another. The mechanism by which iron deficiency produces the above changes is uncertain but is probably associated in part with the structural changes that lead to their microcytic appearance. The altered electrophoretic properties of the transferrin receptor observed in some electrophoretic runs may be a consequence of altered glycosylation.

The observation that Belgrade reticulocyte ghosts contain a protein absent, or present only in very low concentration, in the membrane of heterozygous, iron-deficient, and control reticulocytes is of particular interest. This may be a result of the genetic defect and directly responsible for the disorder of membrane transport of iron. Future work will be directed at studying the nature and function of this protein.

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Analysis With Antiidiotype Antibody of a Patient With Chronic Lymphocytic Leukemia and a Large Cell Lymphoma (Richter's Syndrome)

By L.F. Bertoli, H. Kubagawa, G.V. Borzillo, M. Mayumi, J.T. Prchal, J.F. Kearney, J.R. Durant, and M.D. Cooper

A murine monoclonal antibody made against an idiotypic determinant (Id) of surface IgM/IgD λ molecules on chronic lymphocytic leukemia (CLL) cells of a 71-year-old woman was used for clonal analysis by two-color immunofluorescence. The anti-Id antibody identified IgM⁺/IgD⁺/ λ ⁺ B cells as the predominant cell type of her CLL clone. In addition, substantial proportions of the IgG and IgA B cells and most of the IgM plasma cells in her bone marrow and blood were Id⁺. Six years after diagnosis, the patient died of respiratory failure due to infiltration of lungs by malignant cells. Autopsy revealed a dramatic change in the tumor cell morphology. The lungs, hilar nodes, and liver were infiltrated by a diffuse large cell lymphoma admixed with the

leukemic cells. By immunohistologic staining these anaplastic lymphoma cells were IgM⁺/IgD⁺/ λ ⁺ B cells expressing the same Id noted earlier on the CLL cells. The immunoglobulin gene rearrangement pattern on Southern blot analysis was also the same in leukemic blood cells and in the tissues involved by the lymphoma. Thus, the combination of antiidiotype and immunoglobulin gene analyses in this patient with Richter's syndrome revealed that a CLL clone, seemingly "frozen" in differentiation, was actually undergoing isotype switching, differentiation into plasma cells, and evolution into a rapidly growing and fatal lymphoma.

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PATIENTS with B cell chronic lymphocytic leukemia (CLL) are at risk for the development of polymorphous leukemias, acute lymphoblastic leukemia, or large cell diffuse lymphoma.¹⁻¹⁶ Richter, who described the latter syndrome in 1928, wondered if the lymphoma was related to or independent of the leukemia, noting that the histology of the two lesions did not give "any indication that they are genetically related."¹ Later investigations of Richter's syndrome compared the isotypes of the immunoglobulin (Ig) expressed by the B cells of the lymphoma and the CLL in Richter's syndrome.^{5,6,10,13-16} Often, the two cell types expressed the same Ig isotypes, an event that could have been due to chance alone. Sometimes the heavy chain isotypes differed, an observation that could be explained either by development of a second neoplastic clone or by isotype switching within a single neoplastic clone, as has been observed in multiple myeloma.¹⁷⁻²⁰ Patients with Richter's syndrome in whom the lymphoma and leukemia expressed different light chains^{6,16} can be most easily explained as two independent malignancies.

More reliable clonal markers exist in the variable (V) domains of the Ig molecule and the gene segments that encode them. During the differentiation of pre-B cells, genes of the heavy and then light chain V regions undergo sequential rearrangements that will endow their progeny B cells with distinctive V_H and V_L regions that serve as antigen receptors.²¹ As a result, a clone of malignant B cells has a characteristic rearrangement pattern of Ig genes that can be revealed by Southern blot analysis and unique idiotypic determinants (Id) in the V_H and V_L domains that can be identified by antiidiotype antibodies. These two approaches to clonal analysis of B cell malignancies complement each other. The Southern blot analyzes populations of cells whereas the anti-Id antibody identifies individual cells belonging to the malignant clone, thereby allowing a precise assessment of their morphologic appearance and coexpression of other determinants. Anti-Id reagents have been used to study the cellular origins and differentiation of various types of human B cell malignancies²²⁻²⁸ but use of such antibodies to analyze Richter's syndrome has not been reported. Southern blot analysis in one patient with Richter's syndrome disclosed that the lymphoma represented the emergence of a clone apparently unrelated to the CLL.¹⁶

The current study is the first report of clonal analysis of a patient with Richter's syndrome using an antiidiotype antibody and Ig gene probes. We used these highly specific probes to define the types of cells comprising the CLL clone and the relationship between the CLL and an emerging large-cell lymphoma.

MATERIALS AND METHODS

Patient G.B. G.B., a 71-year-old woman, developed CLL in 1976. Treatment with chlorambucil and prednisone was initially effective, but by January 1982, the disease was refractory and chemotherapy was stopped. By August 1982, she had developed low-grade fever, night sweats, shortness of breath, progressive enlargement of lymph nodes, spleen, and liver, and bilateral pulmonary infiltrates causing respiratory failure. Transbronchial biopsy revealed infiltration of the lung by CLL cells and a few larger blastic cells. Her WBC count at the time of immunofluorescence analysis was 340,000/ μ L (93% lymphocytes, 6% neutrophils, 1% blast), hemoglobin 9.6 mg/dL, hematocrit 27%, platelet count 120,000/ μ L, serum IgM 46 mg/dL, IgG 740 mg/dL, and IgA 122 mg/dL. Serum protein electrophoresis did not reveal a paraprotein, but a very small abnormal arc of free λ chains was observed on immunoelectrophoresis of serum and urine samples. Despite an advanced stage of disease, treatment with 926 mg of mouse monoclonal anti-Id antibody

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markedly reduced the tumor burden in the spleen and circulation and, to a lesser extent, in the lungs. Ventilation was improved transiently, but the patient died of respiratory failure after a month of treatment. Patient G.B. and her family gave informed consent to participate in this study, which was approved by the Institutional Review Board of the University of Alabama at Birmingham Hospitals.

Monoclonal anti-Id antibody. The anti-Id antibody was prepared as previously described.²⁶ In brief, peripheral blood mononuclear cells (MNCs) from patient G.B., most of which were IgM/IgD λ leukemic B cells, were injected subcutaneously at 3-day intervals in BALB/c mice. Hybrids resulting from the fusion of regional lymph node cells with the mouse variant myeloma cell line P3x63-Ag8.653²⁹ were screened for their antibody specificities by an enzyme-linked immunosorbent assay (ELISA) using a panel of myeloma proteins including the homologous IgM λ .²⁶ The latter IgM λ was derived from somatic heterohybrids (GB-Ag) between the patient's leukemic cells and the mouse myeloma cell line; these were constructed as previously described.²⁶ Hybridomas that produced antibodies that reacted with the homologous Ig, but not with other representative myelomas of the same heavy and light chain isotype, were selected and recloned by limiting dilution. One of the clones with anti-Id specificity, GB.8.1, was injected into pristane-primed BALB/c mice to induce ascitic fluid. After dialysis, hybridoma antibody in mouse ascites was purified by diethylaminoethanol (DEAE)-Sephacel ion-exchange column chromatography (Pharmacia Fine Chemicals AB, Uppsala, Sweden) using a continuous ionic gradient (10 mmol/L of Tris-HCl buffer, pH 8.0, to 0.3 mol/L of NaCl-10 mmol/L of Tris HCl buffer, pH 8.0).

Immunofluorescent analysis of cells. Mononuclear cells were isolated from heparinized blood and bone marrow aspirates by Ficoll-Hypaque density-gradient centrifugation. After incubation for 30 to 60 minutes at 37°C to remove cytophilic Ig, nonadherent cells were examined by immunofluorescence.^{26,30} Two-color immunofluorescence was used to examine viable B cells for cell-surface Id and Ig isotype at 4°C in the presence of 0.2% NaN₃, and to stain simultaneously fixed plasma cells for cytoplasmic Id and Ig isotype. This was done using: (a) mouse monoclonal GB.8.1 anti-Id antibody (purified by ion-exchange chromatography); (b) rhodamine-labeled, affinity-purified goat antibody to mouse Ig as a second reagent (this was preabsorbed to remove antibodies cross reactive with human Ig); (c) fluorescein-labeled, affinity-purified goat antibodies to human Ig isotypes as counterstaining reagents (preabsorbed to remove cross-reactivity with mouse Ig).^{26,30} Commercially available antibodies against T cells (Leu 1-5, Becton Dickinson Monoclonal Center, Mountain View, CA), myelomonocytic cells (MMA³¹), natural killer cells (HNK-1³²), B cells (HB-2³³), common acute lymphoblastic leukemia antigen (J5) and HLA-DR antigen (Becton Dickinson Monoclonal Center) were also used.

Immunohistologic analysis of tissues. Tissues obtained at autopsy and fixed in absolute ethanol were embedded in low-melting-point paraffin. Sections were incubated in 10% H₂O₂ in methanol to quench endogenous peroxidase, in 1% bovine serum albumin (BSA) to block nonspecific binding and in phosphate-buffered saline (PBS) for washing. Sections were stained with mouse monoclonal anti-human μ , δ , λ , κ , or GB.8.1 anti-Id followed by biotin-labeled, goat-anti-mouse Ig (preabsorbed to remove antibodies cross-reactive with human Ig) (Southern Biotechnology Associates, Birmingham, AL) and avidin-biotin-horseradish peroxidase complexes (Vector Laboratories, Burlingame, CA). Color was developed with diaminobenzidine, and cells were counterstained with hematoxylin.

Southern blot analysis of peripheral blood and tissues. DNA was extracted³⁴ from blood MNCs frozen at -135°C and from minced tissues that had been obtained at autopsy and fixed in

absolute ethanol. Approximately 10 μ g of DNA from each sample was digested to completion with *Bam*HI (Bethesda Research Laboratories, Gaithersburg, MD) and electrophoresed at 2 V/cm for 60 hours in an International Biotechnologies, Inc. (New Haven, CT) model HRH gel apparatus. The separated DNA fragments were blotted to Zetabind membranes as recommended by the manufacturer (AMF, Meriden, CT). Hybridizations were performed according to Zetabind specifications using J_H 3.2, a 3.2-kilobase (kb) *Bgl*II insert spanning the human J_H exons.³⁵

RESULTS

Specificity of monoclonal anti-Id (GB) antibody. The GB.8.1, a γ 1 κ antibody, proved specific for an Id of the IgM λ molecules expressed by the leukemia cells of patient G.B., as determined by ELISA and immunofluorescence assays. By ELISA, the GB.8.1 antibody reacted with the IgM λ secreted by somatic heterohybrids of the patient's tumor cells and not with a panel of 10 human myeloma proteins and normal IgG. When the somatic heterohybrid cells were stained with anti-Id, anti- μ or anti- λ antibodies, ~60% and ~20% of the cells were μ^+ and λ^+ , respectively. All λ^+ cells coexpressed μ chains. The frequency of Id⁺ cells paralleled that of cells expressing both μ heavy and λ light chains, suggesting that the GB.8.1 anti-Id did not recognize the idiotypic determinants on free μ heavy chains.

The GB.8.1 antibody was found to react with ~1% of B cells and plasma cells from normal individuals, a result that is in agreement with the previous findings in our laboratory and by others.^{25,26,36,37} The GB.8.1 anti-Id antibody stained normal IgM, IgG, and IgA plasma cells with similar frequencies. However, its reactivity was restricted to plasma cells expressing λ light chains, indicating a λ -dependent idiotype.

Expression of surface markers, Ig isotypes, and GB.8.1 idiotype by blood and bone marrow MNCs from patient G.B. Analysis of the phenotype of blood MNCs (without depletion of adherent cells) obtained from patient G.B. before immunotherapy revealed that ~80% expressed the B cell antigen (HB-2), HLA-DR, and the Leu-1 antigen, but < 0.5% were common acute lymphoblastic leukemia antigen (CALLA) positive. The frequencies of T cells and macrophage/monocytes identified by the Leu-4 and MMA antibodies were only 2%, but their absolute numbers in her blood were twofold to fourfold higher than normal values. An inverted ratio of helper T cells (Leu-3⁺, 0.8%) to suppressor or cytotoxic T cells (Leu-2⁺, 1.8%) was also noted. Granular lymphocytes reactive with the HNK-1 antibody were not observed in her blood.

To examine the leukemic cells from G.B. for the expression of Ig isotypes and the GB.8.1 idiotype, two-color immunofluorescence analysis was performed on both blood and bone marrow samples. Eighty percent to 95% of the bone marrow and blood MNCs expressed IgM, IgD and λ determinants on their cell surface (Table 1). Almost all IgM⁺/IgD⁺ cells expressed the GB.8.1 Id, indicating a monoclonal cell origin. Moreover, mature plasma cells expressing IgM λ and GB.8.1 idiotype were demonstrated in both blood and bone marrow samples, suggesting differentiation of some members of the leukemic B cells into IgM λ plasma cells.

Table 1. Two-Color Immunofluorescence Analysis of Ig Isotype and Idiotype Expression by the MNCs of Patient G.B.

Cell Phenotype	Blood		Bone Marrow	
	% MNC (%Id ⁺)		% MNC (%Id ⁺)	
Pre-B				
sig ⁻ cμ ⁺	< 0.05		< 0.05	
B cells				
sigM	97	(> 99)	80	(> 99)
sigD	95	(> 99)	80	(> 99)
sigG	0.05	(77)	NT	
sigA	0.03	(38)	NT	
sκ	< 0.05	(< 10)	NT	
sλ	91	(> 99)	NT	
Plasma cells				
cIgM	0.07	(> 99)	0.05	(87)
cIgG	< 0.05		0.05	(3)
cIgA	< 0.05		< 0.05	(10)
cκ	< 0.05		NT	
cλ	0.05	(> 99)	0.10	(86)

WBC count was 340,000/μL (93% lymphocytes). Eighty-six IgG⁺, 40 IgM⁺, and 40 IgA⁺ bone marrow plasma cells were counted, and 48 IgG and 50 IgA blood B cells were counted.

Abbreviations: s, surface; c, cytoplasmic; NT, not tested.

Circulating B cells expressing other Ig isotypes were extremely low in relative frequencies, but normal or slightly high in absolute numbers. High proportions of both IgG and IgA B cells expressed the GB.8.1 idiotype. Evidence for non-IgM/IgD members of the leukemic clone was also obtained from the idiotypic analysis of marrow plasma cells. A similar distribution of GB.8.1 idiotype among B cells and plasma cells was also observed in the patient's blood 1 year earlier (data not shown). Cells of pre-B phenotype were very infrequent in her marrow and blood samples.

Tumor morphology and distribution at autopsy. After a 30-day course of anti-Id therapy, G.B. died of respiratory failure. The autopsy, performed 3 hours after death, revealed extensive leukemic infiltration of bone marrow, lymph nodes (hilar and retroperitoneal), spleen (950 g), liver (3,000 g), lungs (right 1,350 g; left 1,050 g) and other organs (pancreas, adrenal glands, kidneys, urinary bladder, brain). In addition, hilar lymph nodes, large areas of the lungs, and the hepatic portal triads were diffusely infiltrated by large pleomorphic cells in which the cytoplasm was pale and moderately abundant. The nuclei were often multiple and angular, and the nucleoli were prominent. These large cells were morphologically distinct from the admixed CLL cells, resembling the large cell lymphoma described by Richter in CLL, in which many cells were anaplastic and multinucleated.¹ There were also histologic features of adult respiratory distress syndrome with extensive alveolar hemorrhage and acute renal tubular necrosis.

Expression of Ig isotype and GB.8.1 idiotype by large cell lymphoma. Ethanol fixed sections of the spleen, liver, lungs, and hilar lymph nodes were examined immunohistologically for expression of immunoglobulin determinants (Fig 1). The large, anaplastic lymphoma cells expressed abundant IgM, λ light chains and the GB.8.1 idiotype in their cytoplasm. They did not express IgD, and staining with anti-human κ was negative. Intact membrane immunoglobu-

lin determinants on the small CLL cells were below the threshold of detection in the paraffin-embedded tissues, but immunofluorescence examination of frozen sections of spleen, lymph nodes, and liver showed the CLL cells to be IgM⁺/IgD⁺/λ⁺ and Id⁺.

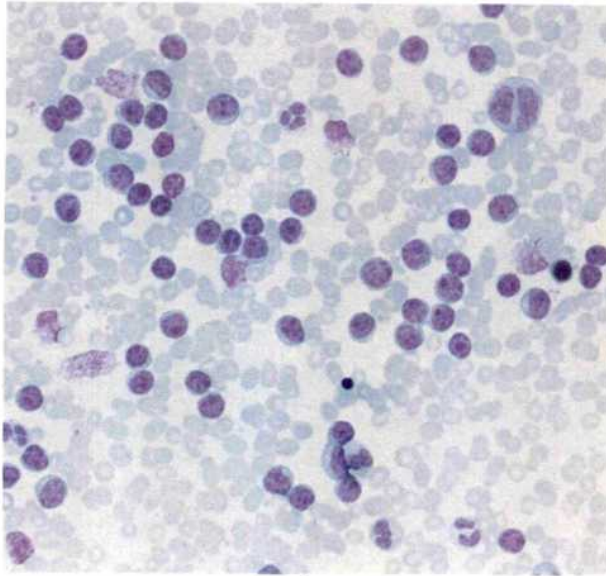
Southern blot analysis of peripheral blood and tissues. The DNA isolated from peripheral blood, in which 97% of the cells were CLL cells, and from the spleen, which was extensively infiltrated by the CLL, was found in both cases to have two prominent J_H segment rearrangements (Fig 2). In the DNA from lung and liver, which were infiltrated by both the CLL and the large cell lymphoma but in which infiltration by malignant cells was less extensive, the J_H segment was predominantly in germline configuration, but fainter bands were visible that corresponded to the rearrangements observed in the blood and spleen samples.

DISCUSSION

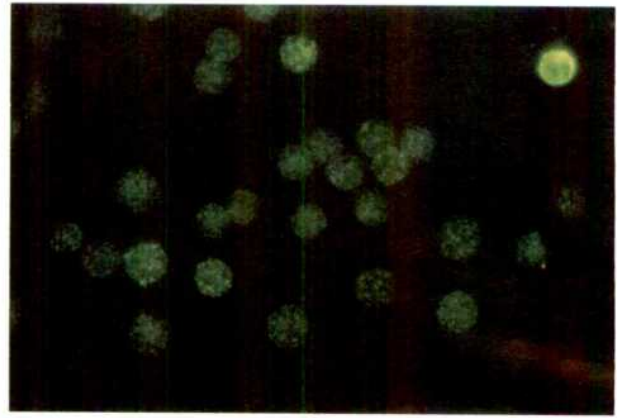
These studies of a patient (G.B.) with CLL, who later developed a large cell lymphoma, provided results that are of interest from several viewpoints. First, although the predominant leukemic cell was an IgM/IgDλ B cell, Id⁺ members of the malignant clone also included IgG and IgA B cells that did not exhibit neoplastic growth characteristics. Small subpopulations of B cells expressing heavy chain isotypes different from the isotype of the predominant malignant cells have also been observed in multiple myeloma, Waldenström's macroglobulinemia, and acute B cell leukemia.^{25-26,28} Similarly, patients have been described with multiple myeloma producing two paraproteins that express the same variable regions but different heavy chain isotypes.¹⁷⁻²⁰ These data suggest that these malignant clones include a preneoplastic precursor cell capable of isotype switching.

IgM plasma cell members of the malignant clone far outnumbered the normal Id⁻ IgM plasma cells, and Id⁺ IgG and IgA plasma cells were present in much lower numbers. These results indicate that differentiation into mature plasma cells was occurring in the leukemic clone although most of the neoplastic cells appeared to have been arrested at the B lymphocyte level. This phenomenon has been observed in an occasional CLL patient with prominent paraprotein derived from plasma cells belonging to the neoplastic clone.³⁸ G.B. did not have such an obvious paraprotein but, using the monoclonal anti-Id antibody in an inhibition radioimmunoassay, circulating Id⁺ immunoglobulin could be demonstrated in a serum concentration of 380 μg/mL (data not shown). Patients with CLL often have a small amount of monoclonal protein in their sera.^{39,40} Together, these data suggest that differentiation to plasma cells by some members of CLL B cell clones is common.

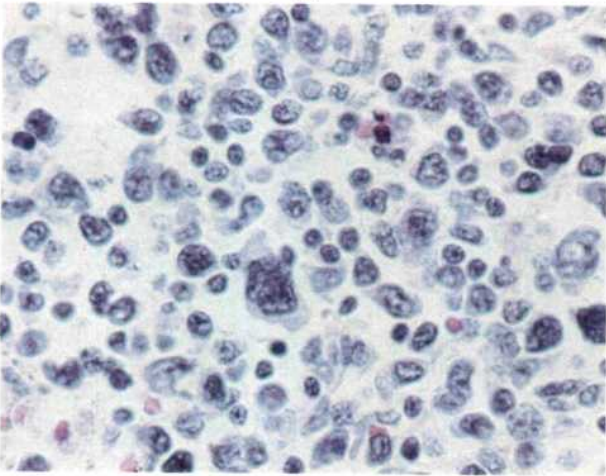
Autopsy revealed that the lungs, hilar lymph nodes, and liver were infiltrated by two populations of tumor cells dramatically different from each other in morphology. One population was constituted by small CLL cells and the other by large anaplastic cells with the histologic appearance of a diffuse large cell lymphoma. Both cell types expressed the same GB.8.1 idiotype. Thus, in this case of Richter's syndrome, the rapidly growing lymphoma must have evolved from the more indolent leukemia. The results of the Southern



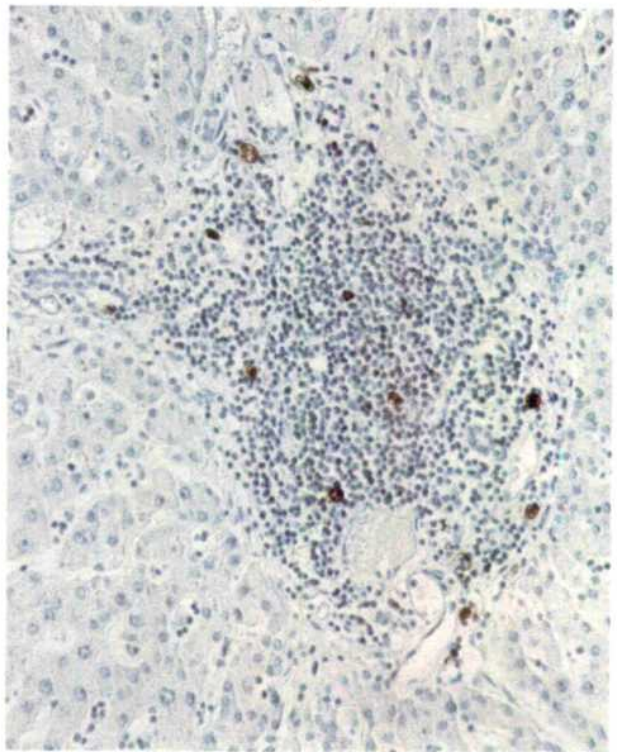
A



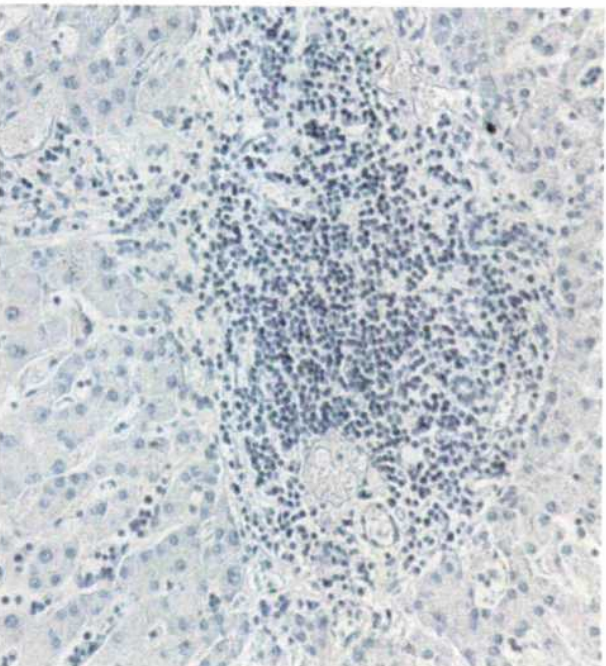
B



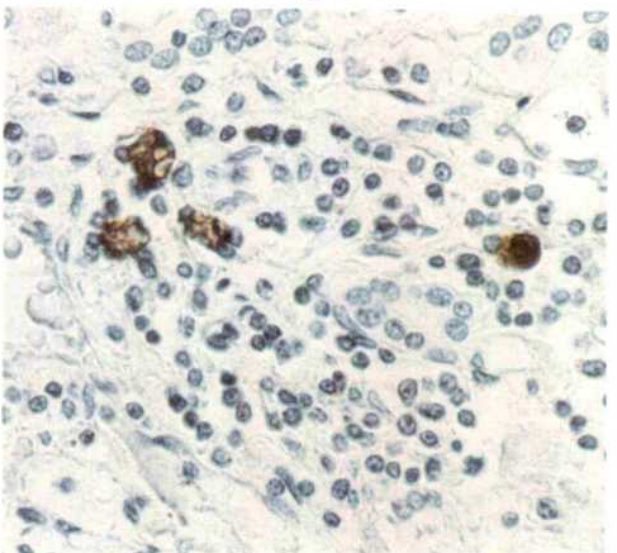
C



D



E



F

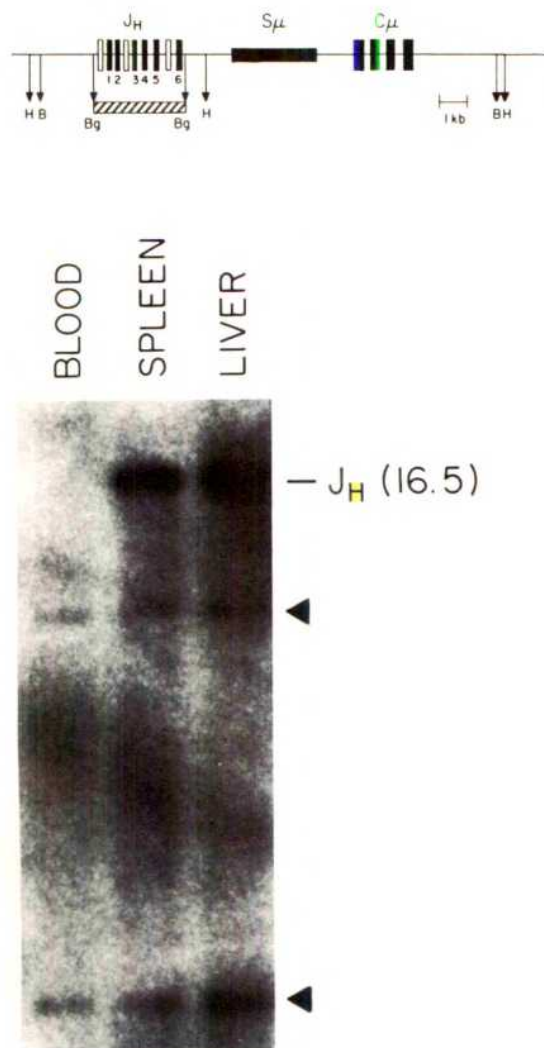


Fig 2. Southern blot analysis of peripheral blood, spleen, and liver cells of patient G.B. Top, restriction enzyme map of a 16.5-kb region spanning the human germline J_H - C_μ region. The J_H probe is a 3.2-kb *Bgl*II (*Bg*) fragment; *B*, *Bam*HI; *H*, *Hind*III. DNA from the tissue samples was digested with *Bam*HI, electrophoresed, blotted, and analyzed with the J_H probe. The blood, spleen, and liver samples contain the same J_H rearrangements (arrowheads), suggesting infiltration of these tissues by a single B cell clone. The same two J_H rearrangements were also found in DNA from lung tissue (data not shown).

blot analysis are consistent with this conclusion because the DNA from tissues infiltrated by both lymphoma and leukemia carried the same J_H rearrangements as the DNA from blood cells in which only the leukemic cells were present. This mutation probably occurred spontaneously or as a result of alkylator therapy before the anti-Id was given, but we

cannot exclude the possibility that growth of the lymphoma accelerated during anti-Id therapy. The lymphoma cells expressed the GB IgM λ but not the IgD λ , and many of them expressed relatively abundant cytoplasmic Ig. Loss of IgD occurs when mature B cells are activated by antigen and growth factors and undergo differentiation to express greater amounts of cytoplasmic Ig.⁴¹ The transformation of the leukemic B cells into anaplastic lymphoma cells may thus mimic the normal pathway of B cell development.

In patients with low-grade B cell lymphomas, the development of high-grade lymphomas has been caused either by a new malignancy or by evolution of the older malignancy.⁴² Potentially, bclonal B cell lymphomas could arise from a common malignant B cell precursor.⁴³ High-grade lymphomas occur more rarely in CLL than in the low-grade lymphomas, and it is not known which mechanism is the more common cause of Richter's syndrome. In one patient, a CLL clone transformed into an acute lymphoblastic leukemia (ALL) expressing Ig with the same antigen binding specificity.² Development of a second independent B cell malignancy has been described in two patients with Richter's syndrome.^{6,16} Richter's syndrome occurring within the same B cell clone has been well documented only in the case of G.B.

Anti-Id antibodies, by providing a precise means of identifying individual cell members of a neoplastic clone, may thus reveal a dynamic picture of a CLL clone undergoing isotype switching, differentiation, and multiple transformational events.

ACKNOWLEDGMENT

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Fig 1. Immunofluorescence and immunohistological analyses of idiotype positive (Id⁺) cells in blood and other tissues from patient G.B. (A) Wright's stain of peripheral blood showing chronic lymphocytic leukemia (CLL) cells. (B) The GB.8.1 idiotype expressed on the surface of CLL cells and in the cytoplasm of a plasmacyte (inset) in peripheral blood. The viable cells (for surface Ig) and the fixed cells (for cytoplasmic Ig) were stained with GB.8.1 anti-Id and then with fluorescein-labeled goat anti-mouse Ig antibodies. (C) Hematoxylin and eosin stain of hilar lymph node section showing a diffuse large cell lymphoma with anaplastic cells. (D through F). Large lymphoma cells in portal triad of liver expressing the GB.8.1 idiotype in their cytoplasm. Liver specimens, fixed in 95% ethanol and embedded in soft paraffin, were stained first with GB.8.1 anti-Id (D, F) or a control antibody (E), then with biotin-labeled goat anti-mouse Ig, and finally with biotin-avidin-horseradish peroxidase complex. The strongly Id⁺ large lymphoma cells are admixed with small CLL cells, the surface idiotype of which is not detectable by this method.

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Clinical Significance of Low Levels of Myeloperoxidase Positivity in Childhood Acute Nonlymphoblastic Leukemia

By Ching-Hon Pui, Frederick G. Behm, David K. Kalwinsky, Sharon B. Murphy, Debra L. Butler, Gary V. Dahl, and Joseph Mirro

The clinical significance of a low percentage of myeloperoxidase-positive blast cells in childhood acute nonlymphoblastic leukemia was determined. Of 155 consecutive cases studied by cytochemical staining methods, 14 were characterized by 4% to 15% (median 6%) myeloperoxidase-positive blasts. All 14 cases showed reactivity to Sudan black B stain, and 7 had Auer rods. The morphological subtypes of leukemia were M1 (8 cases), M2 (3), M4 (1), and M5 (2). Immunological marker studies disclosed the lymphoid-associated T11 antigen on cells from 8 of the 11 cases tested. Other lymphoid-related findings in these 8

cases included the T3 antigen and E rosette formation in 1 case each. Among cases that were prospectively studied for the expression of lymphoid-associated markers, 6 of 8 with low levels of myeloperoxidase positivity compared with only 1 of 44 with higher levels ($>15\%$) possessed such features ($P < 0.001$). We conclude that low levels of myeloperoxidase reactivity distinguish cases of acute leukemia in which the blast cells coexpress lymphoid (T11 antigen) and myeloid markers.

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CLASSIFICATION of the acute leukemias by the French-American-British (FAB) system requires analysis of the cytochemical features of the malignant blast cells.¹ Although reactivity to Sudan black B (SBB) stain can no longer be considered a lineage-specific marker,² myeloperoxidase (MPO) positivity is still regarded to be diagnostic of acute nonlymphoblastic leukemia (ANLL). Investigators in the FAB cooperative group selected $\geq 3\%$ MPO positivity as a criterion for the diagnosis of ANLL.¹ Although the percentage of blasts containing MPO activity ranges widely, from 3% to 100%, most cases of myelocytic, promyelocytic, or myelomonocytic leukemia are characterized by high levels of the enzyme. Whether cases with marginal MPO positivity are sufficiently different from more typical cases to warrant reclassification has not been established. The results reported here indicate that low levels of blast cell MPO activity distinguish a subgroup of children with ANLL whose blasts are more likely to express lymphoid-associated antigens.

MATERIALS AND METHODS

Patients. From April 1980 to April 1986, 155 consecutive children with newly diagnosed ANLL were enrolled in two successive clinical trials at St Jude Children's Research Hospital.^{3,4} Informed consent was obtained for all patients, and the investigations were approved by the institution's clinical trials committee.

Morphological and cytochemical studies. Bone marrow smears were stained by standard techniques, including Wright's-Giemsa, MPO, SBB, periodic acid-Schiff reagent (PAS), naphthol AS-D chloroacetate esterase (CAE) and α -naphthyl butyrate esterase (ANB).⁵ Blast cell morphology was classified according to FAB criteria.¹ By convention, cytochemical staining was considered positive if $>3\%$ of the blasts reacted. Blast cell MPO positivity, based on analysis of 200 cells per sample, was reviewed and confirmed independently by two of us (F.G.B. and D.L.B.). There was good agreement of results between the two readers, with differences generally $<2\%$. In no instance did the discrepancy lead to a different interpretation of MPO positivity.

Immunophenotyping. Leukemic blast cells from marrow aspirates were separated on a density gradient, and only samples containing $>85\%$ blasts were assayed. They were studied for spontaneous rosette formation with use of sheep erythrocytes incubated at 37°C . Cell-surface antigens were detected with a standard indirect immunofluorescence assay using monoclonal antibodies representative of the cluster of differentiation (CD) groups identified at the

First and Second International Workshops on Human Leukocyte Differentiation Antigens. The monoclonal antibodies to myeloid-associated antigens included anti-My-1 (X-Hapten, CD-15),⁶ MCS.1,⁷ MCS.2 (CDw-13, 150),⁸ Mol (CD-11),⁹ and SJ-D1 (CDw-13, 150).¹⁰ The blasts were also tested with monoclonal antibodies to lymphoid-associated antigens, including J5 (common acute lymphoblastic leukemia antigen, CALLA, CD-10),¹¹ T11 (CD-2),¹² T101 (CD-5),¹³ and T3 (CD-3).¹⁴ Two observers independently counted 200 cells in each assay; the results were judged positive if $\geq 40\%$ of the blast cells had surface fluorescence. Terminal deoxynucleotidyl transferase (TdT) was detected by an indirect immunofluorescence assay.¹⁵

RESULTS

MPO activity was demonstrated in 3% to 100% of the blast cells from 37 of 43 cases with an M1 FAB type, 47 of 47 with M2, 9 of 9 with M3, 29 of 29 with M4, and 4 of 25 with M5. The diagnosis of ANLL in the six M1 cases with negative MPO reactivity was based on the presence of Auer rods, ultrastructural myeloperoxidase or myeloid markers as defined by monoclonal antibodies; none of them expressed any lymphoid markers. The two cases of M7 leukemia lacked evidence of the enzyme. Of the 126 cases that showed MPO activity, 14 were characterized by 4% to 15% (median 6%) MPO-positive blast cells in bone marrow samples (Table 1);

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Table 1. Clinical Features and Blast Cell Cytochemical and Immunological Properties Associated with Low MPO Activity

Case	Age (yr)	FAB Type	Auer Rods	Cytochemical Stains					Lymphoid-Associated Markers					
				MPO	SBB	CAE	ANB	PAS	T11 (CD-2)	T101 (CD-5)	T3 (CD-3)	CALLA (CD-10)	TdT	ER
1	13	M4	+	4%	+	+	+	—	+	—	—	—	+	—
2	16	M1	+	4%	+	—	—	+	+	—	+	—	+	—
3	13	M1	+	5%	+	—	—	+	—	—	—	—	+	—
4	17	M1	+	6%	+	+	—	—	+	—	—	—	+	+
5	12	M1	—	6%	+	+	—	—	ND	—	ND	+	—	—
6	6	M1	—	6%	+	+	—	+	+	—	—	+	+	—
7	12	M1	—	6%	+	—	—	+	+	—	—	—	—	—
8	0.4	M2	—	6%	+	+	—	—	ND	ND	ND	ND	—	ND
9	12	M1	—	7%	+	—	—	+	—	—	—	—	+	—
10	7	M5	—	9%	+	+	+	+	ND	ND	ND	ND	ND	ND
11	0.8	M5	—	12%	+	+	+	—	—	—	—	—	—	ND
12	18	M1	+	13%	+	—	—	—	+	—	—	—	+	—
13	14	M2	+	14%	+	—	—	+	+	—	—	—	+	—
14	18	M2	+	15%	+	—	—	—	+	ND	ND	—	ND	—

Abbreviations: ER, E rosette formation at 37°C, ND, not done.

the remaining cases had >25% blast cells with MPO positivity, most >80%. None of the cases had MPO positivity between 16% and 25%. Blast cells from each of these 14 cases with low levels of MPO positivity met standard FAB criteria for ANLL. All cases were positive for SBB, and seven contained Auer rods. Their FAB designations were M1 in 8 cases, M2 in 3, M4 in 1, and M5 in 2.

Blast cells from 12 of the 14 patients (excluding nos. 8 and 10) were examined for lymphoid- and myeloid-associated differentiation markers. All but three cases (nos. 3, 5, and 6) were defined by reactivity to one or more myeloid-associated monoclonal antibodies (data not shown). Studies with lymphoid-associated monoclonal antibodies, although not disclosing a consistent pattern of binding, indicated unequivocal lymphoid characteristics in eight of the 12 cases tested (nos. 1, 2, 4, 6, 7, and 12-14, Table 1). Evidence of lymphoid involvement consisted of reactivity with antibodies to the T11 (CD-2) antigen in all eight cases, coupled with the expression of T3 (CD-3) or formation of heat-stable E rosettes in one case each. Blast cells from an additional patient (no. 5), which were not tested for T11, were positive for CALLA. TdT activity was detected in all but one of the cases that demonstrated T11 and in two others that were devoid of more definitive lymphoid markers.

The relationship between MPO reactivity and the expression of lymphoid-associated antigens was determined in the 56 consecutive patients who had been prospectively studied for these features since August 1983 and had adequate leukemic cell samples. Among these cases, eight (including cases 1, 3, 4, 6, 7, and 11 through 13) were characterized by 4% to 15% MPO-positive blasts, and the remainder were characterized by negative MPO activity (four cases) or >25% MPO positivity (44 cases). Six of eight cases with low MPO reactivity had blast cells with lymphoid-associated markers, contrasted with only one of those with >25% MPO positivity ($p < 0.001$). The patient with >25% MPO also expressed T11 antigen. None of the four cases with negative MPO activity expressed lymphoid-associated antigens.

The clinical features of patients whose leukemic cells

expressed lymphoid-associated markers did not clearly discriminate between these cases and typical ANLL. Ages ranged from 6 to 18 years (median 14 years), presenting leukocyte counts from 2 to $171 \times 10^9/L$ (median $16.2 \times 10^9/L$), platelet counts from 4 to $312 \times 10^9/L$ (median $120 \times 10^9/L$) and hemoglobin levels from 4.1 to 14 g/dL (median 10.4 g/dL). All patients had lymphadenopathy but only three (nos. 1, 2, and 13) had hepatosplenomegaly. Initial CNS leukemia was evident in patient 4, and patient 2 had an anterior mediastinal mass.

DISCUSSION

Bennett and associates¹ considered 3% as the lower limit for MPO positivity in the FAB system of leukemia cell classification. Results of the present study suggest that a higher level of positivity is required to distinguish "typical" cases of ANLL from those with equivocal features. We demonstrated that a criterion of 4% to 15% MPO positivity yields a high percentage of cases with lymphoid-associated differentiation markers, primarily the T11 antigen. Although identified on normal, activated B cells,¹⁶ T11 has not been detected on normal myeloid cells. Additional evidence of lymphoid involvement was provided by T3 positivity and E rosette formation in one case each. Although TdT activity and/or CALLA were also expressed in some of our cases, these markers can no longer be considered lymphoid-specific markers. TdT activity has been demonstrated in 5% to 10% of ANLL cases that were unequivocally myeloid in origin.¹⁷ CALLA has been identified on renal tubular and glomerular cells, granulocytes, fibroblasts, fetal small intestine epithelial cells, mammary epithelium, and myeloma cell lines (see ref. 18 for review). Nonetheless, we think it noteworthy that the majority of our cases with low MPO positivity expressed TdT activity, a far greater proportion than has been reported in ANLL.

The immunologic marker findings in cases with low MPO activity are most compatible with the newly recognized entity of acute mixed-lineage leukemia, in which the leukemic cells coexpress markers generally thought to be

restricted to a single lineage.¹⁹⁻²³ None of the cases in this study had cytogenetic evidence for biclonal disease, whereas evidence for simultaneous expression of lymphoid and myeloid markers was found in all four cases tested (data not shown). The diagnosis of acute mixed-lineage leukemia is best exemplified by case 2. The presence of an anterior mediastinal mass and the expression of T11 and T3 antigens in 83% and 87% of this child's blast cells would be considered by most investigators as diagnostic of T cell acute lymphoblastic leukemia. However, there was also strong evidence for myeloid differentiation, including 4% myeloperoxidase positivity, the presence of Auer rods, and the expression of myeloid antigens in blast cells [84% reacted with Mol (CD-11) and 35% with MCS.1 (CDw-15) monoclonal antibodies].

Consistent with our data, the reported patterns of monoclonal antibody binding in cases of acute mixed-lineage leukemia have generally included high percentages of T11 positivity with variable levels of other lymphoid markers.²¹ These leukemias either may arise from multipotential progenitors with capacity of lymphoid or myeloid differentiation^{22,23} or had aberrant gene expression during malignant proliferation.¹⁹ The clinical importance of acute mixed-lineage leukemia lies in the suggestion that such patients may not respond to therapy conventionally prescribed for myeloid leukemia, but require treatment directed to the lymphoid leukemias.²¹

MPO, a major 135,000-dalton protein, is localized to the

azurophil granules in the myeloid series of hematopoietic cells. MPO synthesis has been suggested to occur at the promyelocytic stage of differentiation and ceases as myeloid cells differentiate to myelocytes, metamyelocytes, granulocytes, and macrophages.²⁴ Abundant MPO activity is typical of progranulocytic leukemia; the activity is decreased in myeloid variants of ANLL and low to absent in monocytic subtypes.²⁵ Thus, the lack of lymphoid markers on blast cells in the single case of M5 leukemia that was tested is not surprising. If acute mixed-lineage leukemia originates in a pluripotent stem cell with the ability to differentiate in either the lymphoid or myeloid pathway,^{19,23} and if the developmental stage of such transformed cells is more primitive than is usually seen in cases of ANLL, one might expect to find low MPO activity as a characteristic feature of this form of the disease.

Currently, the diagnosis of acute mixed-lineage leukemia is based on an extensive battery of cytochemical and immunological tests.²¹ We believe that low MPO positivity in cases of ANLL should be regarded as a potential indicator of this clinical entity. Our interpretation assumes additional significance in view of the variability of clinical and laboratory features among such patients.

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Recombinant Human TNF α Stimulates Production of Granulocyte Colony-Stimulating Factor

By H. Phillip Koeffler, Judith Gasson, John Ranyard, Larry Souza, Michael Shepard, and Reinhold Munker

Tumor necrosis factor α (TNF α) stimulates production of granulocyte colony-stimulating factor (G-CSF) protein and mRNA in fibroblast cells. In contrast, TNF β is at least 1,000-fold less potent than TNF α in increasing levels of G-CSF and GM-CSF activity and mRNA. The fibroblasts

produce G-CSF and GM-CSF mRNA in a coordinate fashion after exposure to TNF. The ability of TNF α to stimulate production of CSF in fibroblasts may be pivotal to regulation of hematopoiesis.

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COLONY-STIMULATING FACTORS (CSFs) are required for the survival, proliferation, and differentiation of hematopoietic cells. The granulocyte (G) and granulocyte-monocyte (GM) CSFs stimulate proliferation of committed stem cells for granulocytes and macrophages, and the GM-CSF can also enhance the proliferation of multipotential, erythroid, eosinophil, megakaryocyte, and mast stem cells.¹⁻⁴ Recent studies suggest that these CSFs also play an important role in the function of mature granulocytes, eosinophils, and monocyte/macrophage.^{1,2,5-7}

Various tissues can produce biologically active CSF, suggesting either that many different cell types can produce CSF or, alternatively that one cell type existing in all tissue may be the source of CSF. Early evidence suggested that monocyte-macrophages were capable of releasing CSF⁸; further studies showed that endothelial cells and fibroblasts,¹⁰ activated T lymphocytes,^{11,12} and cultured nonhematopoietic lines^{13,14} can also produce CSF. Little was known of the mechanism that modulated the production of CSF by the mesenchymal cells until Bagby and co-workers made the seminal observation that conditioned medium from monocytes cultured in vitro stimulated both endothelial cells and fibroblasts to synthesize CSF.^{15,16} Further studies showed that interleukin 1 (IL 1)^{16,17} and tumor necrosis factor (TNF)^{18,19} stimulated endothelial cells, normal fibroblasts, smooth muscle cells, and malignant cells of squamous and adenomatous origin to synthesize GM-CSF protein and mRNA. The present study shows that TNF α stimulates production of G-CSF protein and mRNA in fibroblasts. In contrast, TNF β was nearly 1,000-fold less potent than TNF α in the stimulation of CSF accumulation.

MATERIALS AND METHODS

Fibroblast cultures and preparation of conditioned medium. Normal human adult lung fibroblasts (obtained from informed volunteers A.W. and P.T., by pulmonary lavage) and normal human embryonic lung fibroblasts (WI 38, obtained from the American Type Tissue Culture Collection) were cultured in α medium (Flow Laboratories, McLean, VA) supplemented with 10% fetal bovine serum (FBS). The adult lung fibroblasts were cultured for 6 weeks before their use. By this time the cell population contained no macrophages, as shown by the absence of cells staining for nonspecific esterase. In the experiments described, fibroblasts from the fifth to the fifteenth passage were used, and the cell density ranged from 1 to 5 $\times 10^4$ cells/cm². The CSF-Ca1 line was developed from a patient with adenocarcinoma of the lung. Cells were subcultured by brief (1 minute, 37°C) treatment with 0.05% trypsin/0.02% disodium EDTA (wt/vol in phosphate-buffered saline, PBS). The cells were incubated for periods from 12 hr to 4 d with complete α medium supplemented with varying concentrations of recombinant (r) TNF α , TNF β , or IL 1. Supernatants from

fibroblast cells were prepared by centrifuging the cell culture at 1,000 g for 10 minutes. The conditioned medium (CM) was stored at -20°C for up to 4 months without loss of CSF.

Colony formation in soft agar. Cells were plated in Lux culture dishes in a two-layer soft agar system according to previously described methods.^{20,21} The underlayer contained 0.5% agar; the upper layer contained 0.3% agar (Difco Laboratories, Detroit). The culture medium was α medium. For each plating experiment, 2 $\times$ stock solutions of agar were prepared by autoclaving with distilled water, and the agar was kept at 43°C. For each layer, these stock solutions were mixed with prewarmed α medium [which contained a final concentration of 17% fetal calf serum (FCS) and 1% penicillin-streptomycin], and 1 mL was carefully pipetted into each culture dish. This mixture became semisolid at room temperature within 20 minutes. The CSF and the compounds to be tested were mixed into the underlayer before solidification of the agar. For human bone marrow, 2 $\times 10^5$ cells were plated. Cultures were placed in a humidified atmosphere, 5% CO₂ at 37°C. Colonies (≥ 40 cells) were scored after 10 to 12 days with an inverted microscope. All experiments were done using triplicate or quadruplicate plates for each experimental point. Control plates with no CSF were performed for each experiment.

RNA hybridization studies. For cytoplasmic RNA, freshly harvested cells were suspended in hypotonic buffer 10 mmol/L of Tris-HCl pH 7.4, 1 mmol/L of KCl, 3 mmol/L of MgCl₂ and were lysed with Nonidet P-40 (0.3%). Nuclei were removed by centrifugation. Cytoplasmic RNA was extracted by the phenol/chloroform method as essentially described²² and quantified by absorbance at 260 nm. Samples were denatured at 65°C for 10 minutes, size-separated by an agarose formaldehyde gel [1% agarose (Bethesda Research Laboratories, Gaithersburg, MD), 50 mmol/L of Na Acetate, 10 mmol/L of Na₂ EDTA, 200 mmol/L of MOPS, and 2.2

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mol/L of formaldehyde] and transferred to a nylon-membrane filter (ICN Biomedicals, Irvine, CA). Filters were dried, baked at 80°C in vacuo for 2 hours and then prehybridized for 16 to 24 hours. Hybridizations with [³²P]-labeled cDNAs (1×10^6 cpm/mL) were performed at 42°C for 16 to 24 hours in a solution containing 50% (vol/vol) formamide, $2 \times$ SSC ($1 \times$ SSC is 300 mmol/L of NaCl, 30 mmol/L of sodium citrate), $5 \times$ Denhardt's ($1 \times$ Denhardt's is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin BSA), 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L of EDTA, 10% (vol/vol) dextran sulfate (Sigma) (mol wt 500,000), and 100 µg/mL of salmon sperm DNA (Sigma Chemicals, St Louis). Filters were washed to a stringency of $0.1 \times$ SSC, 1% SDS at 65°C and exposed for 24 to 48 hours at -70°C to Kodak XAR-5 film. The GM-CSF probe was a full-length cDNA²³ that was [³²P]-labeled by nick-translation.²⁴ The G-CSF probe was an oligonucleotide (30 bases) that was end-labeled with [³²P]-γ-ATP.²⁴ The sequence of the oligonucleotide was 5'GGGCTGGGCA-AGGTGGCGTAGAACGCGTA3'. A densitometer was used to scan autoradiograms of the hybridized blots.

Reagents. The TNFα monoclonal antibody was a generous gift of C. Nelson (Genentech). The antibody had a neutralization titer of 6,000 U of TNFα/µg protein. The antibody against GM-CSF is a heterologous rabbit antiserum raised against purified biosynthetic (recombinant, r) GM-CSF; the antibody against G-CSF is a monoclonal antibody raised against rG-CSF. The human TNFα and β and IL 1 were purified to homogeneity and had a specific activity of 3.4×10^7 U/mg, 4×10^7 U/mg, and 3×10^7 U/mg, respectively. With the limulus amoebocyte lysate (LAL) assay, the level of endotoxin detected in the three cytokines was <0.8 ng/mL, which is a level incapable of stimulating GM-CSF mRNA in fibroblasts.

RESULTS

Effect of monokines on CSF synthesis. rTNFα, TNFβ, or IL 1 at various concentrations were added to normal adult lung fibroblasts for 3 days, and the supernatants were harvested and tested for CSF activity using normal human marrow (Fig 1). Both rTNFα and IL 1 stimulated, in parallel, normal human lung fibroblasts in a dose-response fashion to produce increasing amounts of CSF, with 25 and 40 U/mL IL 1 and TNFα, respectively, stimulating 50% maximal levels of CSF. Maximal stimulation by both cytokines occurred at 500 U/mL. In contrast, TNFβ at 5,000 U/mL stimulated fibroblasts only weakly to produce CSF (Fig 1). The highest concentration of IL 1, TNFα, and TNFβ in the supernatants was 500 U/mL. When this concentration was added to soft agar plates containing rGM-CSF (100 pmol/L), IL 1 and TNFβ had no effect, and TNFα inhibited by 20% the clonal growth of GM-CSF in soft agar plates (data not shown).

Experiments were designed to determine how long fibroblasts could synthesize CSF after an initial stimulation with TNFα. The adult lung fibroblasts were incubated with TNFα (300 U/mL) for 72 hours washed, and reincubated for four sequential 24-hour periods in culture medium lacking TNFα (Fig 2). A gradual reduction in CSF release occurred; the cells released 20% of the CSF activity at 72 hours after the TNFα was removed as compared with that produced on the day the TNF was removed from the culture media (Fig 2).

Supernatants from lung fibroblasts cultured for 2 days with TNFα (200 U/mL) were cocultured for 1 hour with a

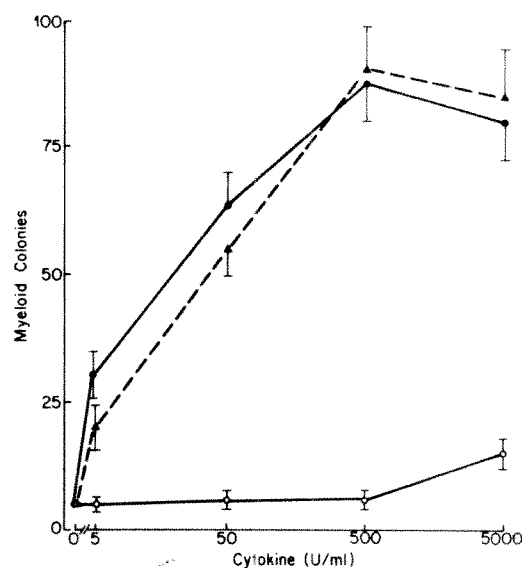


Fig 1. Stimulation of colony-stimulating factor (CSF) production by human fibroblasts cultured with either recombinant tumor necrosis factors TNFα, TNFβ, or interleukin 1 (IL 1). Monolayers of human adult lung fibroblasts were cultured with 0 to 5,000 U/mL of either TNFα (▲—▲), TNFβ (○—○), or IL 1 (●—●) for 4 days. The conditioned media (10% vol/vol) were added to soft agar dishes containing normal human marrow cells (triplicate dishes per TNF concentration). Colonies of ≥ 40 cells were enumerated on day 12 of culture. Results represent mean $\pm$ SE of three experiments.

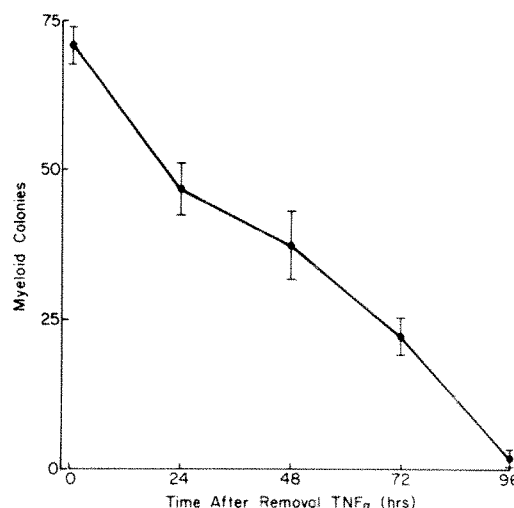


Fig 2. Requirement for the presence of tumor necrosis factor (TNF) α for the production of colony-stimulating factor (CSF) by fibroblasts. Adult lung fibroblasts were cultured for 48 hours in TNFα (200 U/mL) and conditioned media (CM) were harvested and assayed for CSF (represents time 0). The fibroblast cells were washed and reincubated in culture media not containing TNFα. CM were harvested completely at 24-hour intervals and assayed for CSF (10% vol/vol of CM in soft gel culture). The mean $\pm$ SE of three experiments with quadruplicate plates at each time point is shown. Data are presented as a percentage of colonies present at time 0 (mean $85 \pm 8 \pm$ SE).

monoclonal antibody directed against human G-CSF and CSF activity was measured. The G-CSF antiserum decreased the CSF activity of the conditioned medium by a mean 41% as compared with preimmune mouse sera (Table 1). Heterologous rabbit antisera raised against purified rGM-CSF decreased CSF activity by 72% as compared with preimmune rabbit sera. Further experiments showed that G-CSF antibody inhibited rG-CSF but not rGM-CSF, and GM-CSF antibody inhibited rGM-CSF but not rG-CSF (Table 1).

As further proof that TNF was the factor that stimulated CSF production, studies were conducted with a monoclonal antibody to TNF. This antibody (vol/vol) blocked the stimulation of fibroblasts to synthesize CSF in a 1-hour preincubation with rTNF (Table 1). To show that the presence of the anti-TNF did not directly affect the colony assay, the same amount of anti-TNF was added together with rGM-CSF to the soft gel assay (Table 1). Anti-TNF had no effect on colony formation. Likewise, anti-TNF had no effect on the production of CSF by the fibroblast monolayers previously stimulated with TNF α (200 U/mL) (data not shown).

Elevated levels of G-CSF mRNA after exposure of fibroblasts to TNF α . Primary adult lung fibroblasts cultured with TNF α (200 U/mL) contained elevated levels of both G-CSF and GM-CSF mRNA (Figs 3A and B). Stimulation occurred within 24 hours of exposure and increased in activity for the next several days. Messenger RNA for GM-CSF but not G-CSF would be identified in human T lymphotropic virus-1 (HTLV-1) infected T lymphocytes

Table 1. CSF Production After Exposure of Lung Fibroblasts to TNF α

Growth Factor	Fibroblasts Cultured With TNF (200 U/mL)	Antibody	Normal Human Myeloid Colony Formation (Mean $\pm$ SE)
CM fibroblasts	0	0	<10
CM fibroblasts	+	0	82 $\pm$ 7
CM fibroblasts	+	G-CSF*	48 $\pm$ 5
CM fibroblasts	+	GM-CSF*	23 $\pm$ 6
rG-CSF (20 ng)	0	0	78 $\pm$ 8
rGM-CSF (100 pmol/L)	0	0	83 $\pm$ 6
rG-CSF (20 ng)	0	G-CSF	<10
rG-CSF (20 ng)	0	GM-CSF	74 $\pm$ 8
rGM-CSF (100 pmol/L)	0	GM-CSF	<10
rGM-CSF (100 pmol/L)	0	G-CSF	79 $\pm$ 7
CM fibroblasts	+	TNF*	<5
rGM-CSF (100 pmol/L)	0	TNF*	79 $\pm$ 7

TNF (200 U/mL) was cultured with adult lung fibroblast cells (5×10^5 /mL) for 3 days in the presence of α medium plus 10% fetal calf serum. CM were collected and tested (10% vol/vol) for their ability to stimulate clonal proliferation of normal human marrow myeloid stem cells. Results represent mean of two experiments with quadruplicate plates.

*The G-CSF monoclonal antibody and the GM-CSF heterologous antisera raised in rabbits against human rGM-CSF were precultured (vol/vol) with the CM for 1 hour at 23°C and then added to culture dishes to test for CSF activity. TNF monoclonal antibody was precultured (vol/vol) with TNF for 1 hour at 23°C and then added to culture dishes to test the ability of fibroblasts to be stimulated to synthesize CSF.



Fig 3. (A and B) Effect of tumor necrosis factors TNF α and TNF β on granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage (GM)-CSF mRNA levels in adult lung fibroblasts. Cytoplasmic RNA (15 μ g/lane) was prepared and analyzed by formaldehyde-agarose gel electrophoresis and nylon-base paper blotting as previously described.²⁴ Lanes 1 through 5, days 0 through 4 exposure of fibroblasts to TNF α (200 U/mL), respectively; lane 6, HL-60 promyelocytes (do not express detectable CSF); lane 7, HTLV-1 immortalized T lymphocytes (SLB-1). The blot shown in A was probed with G-CSF, and the 1.6-kb hybridizing band is consistent with G-CSF mRNA; the blot in B was probed with GM-CSF, and the 0.9-kb hybridizing band is consistent with GM-mRNA. Autoradiograms were exposed for 2 days. The GM-CSF probe was gel-purified, full-length GM-CSF cDNA¹⁸ that was nick-translated to a specific activity of $>10^8$ cpm per μ g of DNA. The G-CSF is an oligonucleotide probe² that was end-labeled with γ -ATP³²p.²⁴

(SLB-1, lane 7). Levels of both G-CSF and GM-CSF mRNA were coordinately expressed in the adult fibroblasts cultured with TNF α .

We also examined the effect of TNF α on mRNA levels of G-CSF and GM-CSF in human embryonic fibroblasts (WI-38) (Figs 4A and B). These cells, similar to adult fibroblasts, constitutively contain a small amount of G-CSF and GM-CSF mRNA. The G-CSF and GM-CSF mRNA accumulation reached near maximal levels by 4-hour and 1-hour exposure to TNF α (200 U/mL), respectively, and returned toward baseline by the second day of culture.

The effect of TNF β on G-CSF and GM-CSF accumulation in lung fibroblasts was examined. Exposure of the adult fibroblasts to TNF β (200 and 400 U/mL for 2 days) only slightly stimulated levels of G-CSF and GM-CSF mRNA (Fig 5A and B).

DISCUSSION

Bagby and colleagues showed that CSF production by T lymphocytes, endothelial cells, and fibroblasts is stimulated

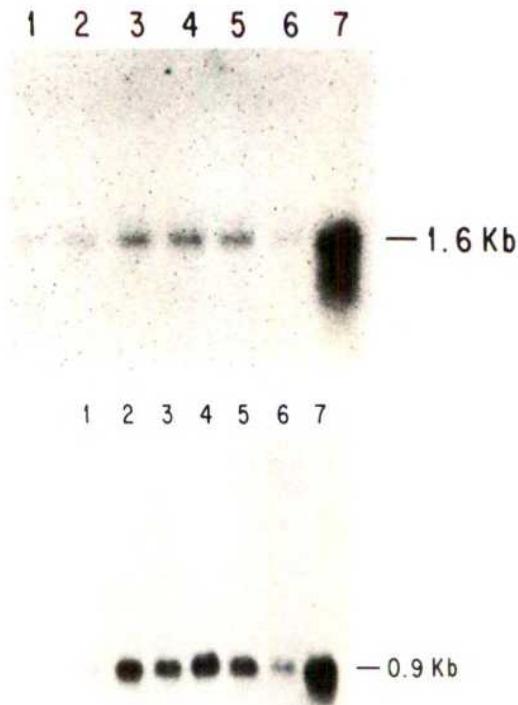


Fig 4. (A and B) Effect of tumor necrosis factor $\text{TNF}\alpha$ on granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage (GM)-CSF mRNA levels in embryonic lung fibroblasts. Embryonic fibroblasts (WI-38) were exposed to $\text{TNF}\alpha$ (200 U/mL) as follows: lane 1, 0 hours; lane 2, 1 hour; lane 3, 4 hours; lane 4, 12 hours; lane 5, 24 hours; lane 6, 48 hours; lane 7 contains RNA from a lung carcinoma cell line that synthesizes CSF (CSF-Ca1). Each lane contains 15 μg of RNA. The 1.6- and 0.9-kb hybridizing bands are consistent with G-mRNA and GM-mRNA, respectively. Autoradiograms were exposed for 2 days. Figure 4A was probed with the G-CSF oligonucleotide; Fig 4B was probed with GM-CSF cDNA. Methods were the same as in Fig 3.

by a soluble monocyte product, which they called monocyte-derived recruiting activity (MRA).^{15,16,25} We previously showed that $\text{TNF}\alpha$ stimulated fibroblasts to produce GM-CSF.¹⁸ The present study shows clearly that fibroblasts also can be induced to produce G-CSF. Likewise, the levels of G-CSF and GM-CSF mRNA markedly increase in the fibroblasts after these cells are exposed to $\text{TNF}\alpha$. Other investigators showed that $\text{TNF}\alpha$ stimulated endothelial cells to produce mRNA that codes for GM-CSF but not G-CSF.¹⁹ Together the findings suggest that not all mesenchymal cells are stimulated by $\text{TNF}\alpha$ to produce G-CSF. Our studies showed that after the fibroblasts were stimulated by $\text{TNF}\alpha$ to synthesize CSF, the cells continued to produce CSF for several days. This may be important in vivo to ensure continued, uninterrupted production of CSF to activate granulocytes and macrophages to contain inflammation.

We showed that $\text{TNF}\beta$ was ~1,000-fold less potent than $\text{TNF}\alpha$ in stimulation of production of CSF by fibroblasts. The $\text{TNF}\beta$ stimulated by less than twofold over baseline the levels of G-CSF and GM-CSF mRNA in fibroblasts as determined by densitometry. In contrast, levels of GM-CSF and G-CSF mRNA increased greater than eight- and three-

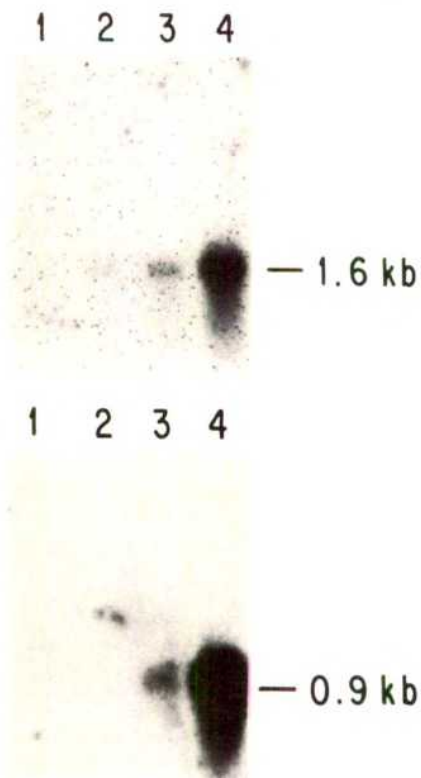


Fig 5. (A and B) Effect of tumor necrosis factor $\text{TNF}\beta$ on granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage (GM)-CSF mRNA levels in adult and embryonic lung fibroblasts. Methods were the same as in Fig 3. A and B show RNA from adult fibroblasts exposed for 3 days as follows: lane 1, 0 $\text{TNF}\beta$; lane 2, 200 U/mL $\text{TNF}\beta$; lane 3, 400 U/mL of $\text{TNF}\beta$; lane 4, CSF-Ca1 lung carcinoma cells that synthesize CSF. A and B were probed with a ^{32}P -labeled G-CSF oligonucleotide and a GM-CSF cDNA, respectively. The 1.6- and 0.9-kb hybridizing bands are consistent with G-mRNA and GM-mRNA, respectively. Autoradiograms were exposed for 7 days. Methods were the same as in Fig 3.

fold, respectively, in fibroblasts cultured with $\text{TNF}\alpha$. This represents the first report to our knowledge in which the two TNF proteins did not have nearly identical biologic properties. The two proteins have 30% amino acid homology, and many of the remaining differences between the two polypeptides result from conservative amino acid changes.²⁴ However, amino acids 67-109 of $\text{TNF}\alpha$ are unrelated to the corresponding region of $\text{TNF}\beta$. This apparently unrelated region of $\text{TNF}\alpha$ may specify the differences in their ability to stimulate CSF production by fibroblasts.

One of the major roles of mononuclear phagocytes in the regulation of granulopoiesis may occur through production of $\text{TNF}\alpha$ and IL 1, which recruit fibroblasts and other mesenchymal cells to produce CSF. Besides being necessary for proliferation, differentiation, and survival of hematopoietic progenitor cells, they also enhance a number of functional activities of mature granulocytes and macrophages, such as inhibition of migration, phagocytosis of microbes, oxidative metabolism, and antibody-dependent cytotoxic killing of tumor cells.⁵⁻⁸ Our data demonstrate that $\text{TNF}\alpha$ modulates the levels of both G-CSF and GM-CSF in

fibroblasts, which has several important implications. First, G-CSF and GM-CSF will be synthesized locally at inflammatory sites by activation of tissue macrophages to produce TNF α . This local G-CSF and GM-CSF can activate mature granulocytes and macrophages to migrate to inflammatory foci and, by inhibiting further migration, ensure their retention in the region of inflammation. Second, the G-CSF and GM-CSF produced as a result of TNF synthesis by macrophages at the inflammatory site may enhance proliferation of hematopoietic stem cells, leading to an increased production of the granulocytes and macrophages required to fight infection. In diseases such as rheumatoid arthritis and in

several of the collagen-vascular disorders, inflammation with its increased number of activated macrophages and granulocytes can be deleterious, causing bone and soft tissue damage. In these cases, the ability to decrease either synthesis of TNF α by macrophages or production of G-CSF and GM-CSF by TNF-stimulated mesenchymal cells at the site of inflammation may be therapeutically useful.

ACKNOWLEDGMENT

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Changes in Neutrophil Surface Protein Composition Accompany Phagocytosis

By Keith M. Skubitz and Tracy K. Kinkead

Phagocytosis is a critical host defense mechanism of neutrophils. In this study, membrane protein changes occurring during phagocytosis were studied in human neutrophils using surface radiolabeling before or after phagocytosis of various target particles. Cells were labeled at the cell surface using lactoperoxidase-catalyzed iodination or neuraminidase-galactose oxidase- NaB^3H_4 , galactose oxidase- NaB^3H_4 , or periodate- NaB^3H_4 techniques. Such studies are complicated by the fact that these techniques identify many surface proteins on the phagocyte, and labeling after phagocytosis occurs often results in radiolabeling proteins of the target particle, thus making changes in cell-surface proteins more difficult to detect. Immunoprecipitation with monoclonal antibody AHN-1, which

reacts with a carbohydrate present on several human neutrophil surface proteins and inhibits phagocytosis, eliminated interference caused by radiolabeled proteins of the target particle and simplified analysis by restricting the study to a limited number of proteins. AHN-1 immunoprecipitated less radiolabeled protein from neutrophils labeled after phagocytosis of particles opsonized with IgG or complement than from cells labeled before phagocytosis. Isolation of phagocytic vesicles containing opsonized emulsified paraffin oil demonstrated that three proteins of mol wt 105,000, 140,000, and 170,000 recognized by AHN-1 were internalized in the phagocytic vesicle during phagocytosis.

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THE NEUTROPHIL plays an important role in host defense against bacterial infections. One critical neutrophil host defense mechanism is phagocytosis, a process of envelopment and internalization of extracellular particles. This process can be "nonspecific" (eg, phagocytosis of latex particles) but is most efficient when the target particle is opsonized by IgG or complement. During phagocytosis, the target particle is enveloped by a vesicle derived from the plasma membrane, and large areas of the neutrophil plasma membrane are internalized with the target particles. Membrane and particle internalization occur at localized sites of cell-target contact.

Cell-surface glycoproteins play important roles in cell function and are presumably important in the process of phagocytosis as well. Indeed, neutrophils deficient in certain membrane glycoproteins have phagocytic defects.¹⁻⁴ Membrane protein changes occurring during phagocytosis have been studied in both neutrophils⁵⁻⁸ and macrophages,⁹⁻¹⁴ and many of these studies of membrane protein changes have used surface radiolabeling techniques. These studies have been complicated by the fact that the techniques identify a large number of surface proteins on the phagocyte, and labeling after phagocytosis occurs often results in radiolabeling proteins of the target particle, thus making changes in cell-surface proteins more difficult to detect. We used two different surface-labeling techniques and immunoprecipita-

tion with monoclonal antibody AHN-1 to study the surface protein composition of human neutrophils before and after phagocytosis of four different target particles. The use of immunoprecipitation with a monoclonal antibody specific for the phagocyte eliminated interference caused by radiolabeled proteins derived from the target particle and further simplified the analysis by restricting the number of surface proteins visualized. AHN-1 is an IgM monoclonal antibody that reacts with a carbohydrate epitope present on several different surface proteins of human neutrophils.¹⁵⁻¹⁷ AHN-1 also specifically inhibits phagocytosis by human neutrophils, suggesting that one or more of the surface proteins recognized by AHN-1 may play a role in this process.¹⁸ We found that AHN-1 immunoprecipitated less radiolabeled protein from neutrophils labeled at the cell surface with ^{125}I or ^3H after phagocytosis of particles opsonized with IgG or complement than from cells labeled before phagocytosis of the same target particles. Our results may have been due to internalization of surface proteins, resulting in fewer of these proteins on the surface, or to some other effect that altered the efficiency of protein labeling, such as a conformational change. To characterize these surface protein changes further, we isolated phagocytic vesicles from neutrophils labeled at the cell surface with ^{125}I or ^3H before or after phagocytosis of opsonized emulsified paraffin oil droplets. The proteins recognized by AHN-1 were shown to be internalized during phagocytosis by both radiolabeling techniques. These results demonstrate that these proteins are closely associated with the process of phagocytosis and further characterize their fate during particle ingestion.

MATERIALS AND METHODS

Cell preparation. Normal peripheral blood neutrophils were prepared by a modification of the methods of Boyum and Craddock and colleagues^{19,20} as previously described. Cells were suspended at appropriate concentrations in phosphate-buffered saline (PBS), pH 7.4 or Hanks' balanced salt solution (HBSS), pH 7.4 (GIBCO Laboratories, Grand Island, NY) as indicated. Differential cell counts on Wright's-stained cells routinely revealed >95% neutrophils.

Materials. Anti-human neutrophil monoclonal antibodies AHN-1 and AHN-1.1 were produced as previously described.¹⁵ AHN-1.1 is an IgM monoclonal antibody with a similar affinity for

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lacto-*N*-fucopentaose III (LNF III) as AHN-1.^{16,17} Ascites fluid from mice bearing the appropriate IgM-secreting hybridomas was heat-inactivated at 56°C for 30 minutes and then centrifuged at 100,000 g for 1 hour at 4°C prior to use. Antibody 60.3, a CDw18 monoclonal antibody,² was a gift of Drs P. Beatty and J. Harlan (University of Washington, Seattle).

Sheep erythrocytes sensitized with rabbit IgG (EA7S), sheep erythrocytes sensitized with rabbit IgG, guinea pig C1, and human C4, C2, and C3 (EAC423), and unopsonized sheep erythrocytes (E) were obtained from Cordis Laboratories, Miami. Before use, sheep erythrocytes were washed once in PBS at 23°C and resuspended at the desired concentration in HBSS containing 0.2% bovine serum albumin (BSA) (Sigma Chemical, St Louis). Latex beads, 2.0 μ m in diameter, were obtained from Polyscience, Warrington, PA. Before use, latex particles were washed once in PBS, incubated for 30 minutes at 23°C in HBSS containing 5% BSA, washed again in PBS, and then suspended at the desired concentration in HBSS containing 0.2% BSA (HBSS-BSA). Zymosan (ICN Pharmaceuticals, Cincinnati) was opsonized with fresh human serum (2 mg/mL) for 30 minutes at 37°C, washed once with PBS, and resuspended in HBSS as described above.

¹²⁵I Radiolabeling. Neutrophils were radiolabeled with ¹²⁵I at the cell surface using lactoperoxidase as previously described^{21,22} with minor modifications. Radiolabeling was performed before or after phagocytosis of various target particles as indicated. In brief, 1 mCi of Na¹²⁵I (100 mCi/mL carrier-free, Amersham, Arlington Heights, IL) was added to 10⁷ neutrophils in 1 mL of PBS containing 20 mmol/L of glucose, 0.22 U of lactoperoxidase (Sigma), and 0.02 U of glucose oxidase type V (Sigma), at 0°C, and the cells were then incubated for 25 minutes at 0°C. The reaction was terminated by adding 10 mL of 0°C PBS and then adding diisopropylfluorophosphate (DFP) (Sigma) to 5 mmol/L. After 10 minutes at 0°C, the cells were washed twice with 10 mL of PBS at 0°C. In some cases, as indicated, cells were washed twice with 10 mL of PBS at 0°C without DFP treatment, resuspended in 100 μ L of HBSS, and mixed with target particles for phagocytosis as described below. In these cases, the cells were treated with DFP after phagocytosis but before solubilization.

NaB³H₄-Radiolabeling. Neutrophils were labeled with ³H at the cell surface using NaB³H₄ as previously described with minor modifications.²³⁻²⁵ Radiolabeling was performed before or after phagocytosis of various target particles as indicated. Three different methods were used. In the first two methods, 2 $\times$ 10⁷ neutrophils were incubated with 5 U of galactose oxidase Type V (E.C. 1.1.3.9.) (Sigma) with or without 1 U of neuraminidase Type X (Sigma) in 1 mL of HBSS at 37°C for 30 minutes. The cells were then washed twice with 1 mL of PBS at 0°C and resuspended in 500 μ L of PBS at 23°C. Two mCi of NaB³H₄ (Specific activity 15 Ci/mmol, Research Products International, Mount Prospect, IL) in 10 μ L of 0.1 mol/L of NaOH was added, and the mixture was then incubated at 23°C for 30 minutes. The cells were washed twice with PBS at 0°C. In the third method, 2 $\times$ 10⁷ neutrophils were incubated in PBS containing 1 mmol/L of NaIO₄ for 10 minutes at 0°C in the dark. The cells were then washed twice with PBS at 0°C, resuspended in 500 μ L of PBS at 23°C and treated with NaB³H₄ as described above. Cells were treated with DFP before solubilization as described for ¹²⁵I-labeling.

Phagocytosis. Phagocytosis of four different target particles was performed before or after radiolabeling, as indicated. Neutrophils, 2 $\times$ 10⁷ in 200 μ L of HBSS, were mixed with 100 μ L of HBSS-BSA containing 2 $\times$ 10⁸ sheep erythrocytes, 2 $\times$ 10⁸ latex particles, or 300 μ g of opsonized zymosan, and the mixture was incubated for 30 minutes at 37°C. When neutrophils were radiolabeled before phagocytosis, phagocytosis was terminated by adding 1

mL of PBS at 0°C and centrifuging at 400 g for 6 minutes. When neutrophils were radiolabeled after phagocytosis, phagocytosis was terminated by adding PBS and DFP as described above.

Preparation of phagocytic vesicles. Phagocytic vesicles of neutrophils labeled at the cell surface were prepared using opsonized Oil Red O particles as previously described²⁶⁻²⁹ with minor modifications. Opsonized Oil Red O-containing particles (ORO) were prepared as described, suspended in 1 mL of HBSS, and aliquots were frozen and stored at -20°C for no longer than 1 month before use.

Phagocytosis was performed in 50-mL polypropylene tubes either before or after radiolabeling. Neutrophils, 3 $\times$ 10⁷/mL, were suspended in 4 mL of HBSS containing 10 mmol/L *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (GIBCO), pH 7.2, and prewarmed for 15 minutes at 37°C. Freshly vortexed prewarmed ORO particles, 1.6 mL, were then added, and the mixture was incubated for 30 minutes at 37°C with frequent mixing. Phagocytosis was stopped by adding 40 mL of 0°C saline containing 1 mmol/L of *N*-ethylmaleamide (Sigma). Uningested ORO particles were then removed by centrifugation at 400 g for 6 minutes at 4°C. The cell pellet was resuspended and washed twice by suspending in 50 mL of PBS at 0°C and centrifuging at 400 g for 6 minutes and then was washed once with 20 mL of homogenization buffer [0.34 mol/L of sucrose containing 0.5 mg/mL BSA, 2 mmol/L of phenylmethylsulfonylfluoride (PMSF) (Sigma), and 10 mmol/L of HEPES, pH 7.2] at 4°C.

Phagocytic vesicles were then prepared by suspending the cells in 0.8 mL of homogenization buffer at 0°C in a Potter-Elvehjem homogenizer with a tight Teflon pestle (Kontes Glass, Vineland, NJ) and homogenized. Homogenization consisted of 10 up-and-down strokes during 1 minute with a Wheaton Overhead stirrer (Wheaton Instruments, Millville, NJ) set at a speed of 4. The homogenate was then centrifuged at 400 g for 6 minutes, and the supernatant was layered over a discontinuous gradient composed of 0.8 mL each of 50%, 45%, and 35% (wt/vol) sucrose containing 0.05 mg/mL of BSA, and 10 mmol/L of HEPES, pH 7.2. The supernate was then overlaid with 8.6% sucrose containing 10 mmol/L of HEPES, pH 7.2, and the samples were then centrifuged at 100,000 g_{av} for 60 minutes at 4°C. The phagocytic vesicles were then collected from the surface, and the plasma membrane fraction was recovered from the interface between the 35% and 45% sucrose layers. The plasma membrane fraction was washed by diluting with 5 vol of 10 mmol/L of HEPES, pH 7.2, containing 2 mmol/L of PMSF and centrifuged at 27,000 g_{av} for 45 minutes at 4°C.

When cells were surface-labeled after phagocytosis, phagocytosis was performed exactly as described above except that following the *N*-ethylmaleamide wash the cells were washed twice with PBS at 0°C and resuspended in the appropriate buffers for radiolabeling as described above. Following radiolabeling, the cells were washed in 20 mL of homogenization buffer, and phagocytic vesicles were prepared as described above.

Immunoprecipitation and polyacrylamide gel electrophoresis. Neutrophils (1 to 2 $\times$ 10⁷) labeled with ¹²⁵I or ³H and treated with DFP as described above were suspended in 1.0 mL of cell solubilization buffer (20 mmol/L of Tris-HCl, pH 7.6, 150 mmol/L of NaCl, 0.5% Nonidet P-40, 0.02% Na₃N, and 2 mmol/L of PMSF) and incubated on ice for 1 hour.¹⁵ Radiolabeled proteins of phagocytic vesicles and plasma membrane fractions were also solubilized in solubilization buffer as described above. The suspensions were then centrifuged at 100,000 g for 1 hour at 4°C. The resulting supernatants were used for immunoprecipitation or analyzed by NaDodSO₄ (SDS-PAGE). Immunoprecipitation was performed as previously described except that extracts were not preabsorbed with *Staphylococcus aureus*.¹⁵ SDS-PAGE and autoradiography were performed as described.^{15,30}

RESULTS

Changes in surface proteins during phagocytosis detected by ^{125}I -labeling. Changes in neutrophil surface proteins occurring during phagocytosis were examined by surface-labeling with ^{125}I using lactoperoxidase, before and after phagocytosis of various target particles. Analysis of radiolabeled proteins in extracts of cells labeled with ^{125}I before and after phagocytosis revealed a complex pattern of many radiolabeled proteins (data not shown). Analysis was further complicated by the appearance of radiolabeled proteins from the target particles. We used immunoprecipitation by the granulocyte-specific monoclonal antibody AHN-1 for better definition of changes in surface proteins occurring during phagocytosis.¹⁵ AHN-1 recognizes a carbohydrate epitope present on several surface proteins of human neutrophils^{16,17} and also inhibits phagocytosis by human neutrophils.¹⁸ AHN-1 immunoprecipitated ^{125}I -labeled proteins of ~105,000 and 135,000 to 190,000 daltons from resting neutrophils (Fig 1, Lane A) as previously described.¹⁵⁻¹⁷ The higher mol wt protein complex migrated as a broad band and was often resolved into two separate proteins with mol wt of ~140,000 and ~170,000 as previously described.¹⁶ To test the effect of the process of phagocytosis on surface proteins previously labeled with ^{125}I , cells were labeled with ^{125}I , washed, and then allowed to phagocytose test particles. The patterns of radiolabeled proteins immunoprecipitated by AHN-1 from cells labeled prior to phagocytosis of EA7S (Fig 1, lane B), EAC423 (Fig 1, lane C), opsonized zymosan (Fig 1, lane D), or latex particles (Fig 1, lane E) were similar to that from cells solubilized prior to phagocytosis (Fig 1, lane A).

In contrast, immunoprecipitates from neutrophils labeled with ^{125}I after phagocytosis were strikingly different. Immunoprecipitates from cells labeled after phagocytosis of EA7S (Fig 1, lane F), EAC423 (Fig 1, lane G), or opsonized zymosan (Fig 1, lane H) contained almost no detectable radiolabeled 140-kd protein (Fig 1, arrow). In some experiments, the intensity of the 105-kd protein decreased as well. Phagocytosis of latex particles (Fig 1, lane I) or unopsonized RBCs (E) (data not shown) before radiolabeling had little effect on the radiolabeled proteins immunoprecipitated by AHN-1. Phagocytosis of opsonized or unopsonized ORO particles before ^{125}I -labeling resulted in a decrease in intensity of both the 140- to 170-kd and 105-kd proteins immunoprecipitated by AHN-1, although this decrease was less marked than that observed with EA7S or EAC423 (data not shown). Normal mouse serum (NMS) did not immunoprecipitate these major radiolabeled proteins from ^{125}I -labeled resting neutrophils (Fig 1, lane N) or any of the other extracts tested (data not shown). Similarly, AHN-1 did not specifically immunoprecipitate any radiolabeled proteins from extracts of target particles labeled with ^{125}I using lactoperoxidase (Fig 1, lanes J through M). Immunoprecipitates using AHN-1.1 were identical to those using AHN-1 (data not shown). Microscopical examination (Table 1) confirmed that neutrophils readily phagocytosed opsonized RBCs but not unopsonized cells. The latex particles were less efficiently phagocytosed. More than 50% of neutrophils

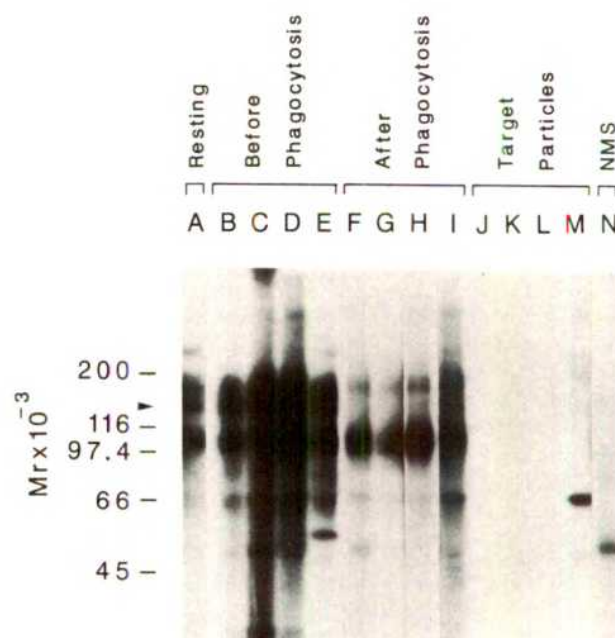


Fig 1. Immunoprecipitation by AHN-1 of radiolabeled proteins of neutrophils labeled with ^{125}I at the cell surface before and after phagocytosis. Neutrophils were surface labeled with ^{125}I using lactoperoxidase before or after phagocytosis of various test particles, solubilized, and immunoprecipitated as described in the text. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 5% to 15% linear gradient polyacrylamide gel and autoradiography as described in the text. Approximately 2×10^6 neutrophil and 2×10^7 RBC equivalents were added to each immunoprecipitation reaction shown in lanes A through I and N. Approximately 2×10^7 RBC equivalents (the same amount present in lanes A through I) were added to each immunoprecipitation reaction shown in lanes J through M. Lanes A through M: immunoprecipitates with monoclonal antibody AHN-1; lane A: ^{125}I labeled resting neutrophils; lanes B through E: cells labeled before phagocytosis; lanes F through I: cells labeled after phagocytosis; and lanes J through M: target particles labeled with ^{125}I . Phagocytic targets were: lanes B, F, and J—EA7S; lanes C, G, and K—EAC423; lanes D, H, and L—opsonized zymosan; and lanes E, I, and M—latex beads coated with BSA. Each extract was also immunoprecipitated with normal mouse serum (NMS). Lane N shows the immunoprecipitate with NMS of the same extract shown in lane B immunoprecipitated with AHN-1. Immunoprecipitates with NMS from the extracts used in lanes A through I appeared similar to lane N. Proteins used as mol-wt standards were myosin heavy chain, 200,000; *Escherichia coli* β -galactosidase, 116,000; phosphorylase B, 97,400; bovine serum albumin, 66,000; and ovalbumin, 45,000. Two replicate experiments gave similar results.

Table 1. Phagocytosis of Target Particles

Target	No. of Target Particles per Neutrophil						
	0	1	2	3	4	5	>5
E	96 $\pm$ 2	4 $\pm$ 2	0	0	0	0	0
EA7S	31 $\pm$ 5	26 $\pm$ 2	20 $\pm$ 3	13 $\pm$ 3	6 $\pm$ 2	3 $\pm$ 1	2 $\pm$ 1
EAC423	17 $\pm$ 1	16 $\pm$ 4	10 $\pm$ 8	16 $\pm$ 3	15 $\pm$ 2	9 $\pm$ 2	12 $\pm$ 2
Latex	50 $\pm$ 4	27 $\pm$ 5	12 $\pm$ 3	7 $\pm$ 3	2 $\pm$ 1	2 $\pm$ 2	1 $\pm$ 1

Phagocytosis was performed as described in the text, and the number of target particles ingested by each neutrophil was determined by light microscopy. Results are reported as the mean $\pm$ SD of three determinations.

(Percentage of Neutrophils Phagocytosing Indicated No. of Target Particles)

incubated with ORO contained identifiable ORO particles of various sizes (data not shown).

Because neutrophils contain enzyme systems capable of halogenating proteins,^{6,31} we performed control experiments in which Na^{125}I was added to the labeling mixture but lactoperoxidase was omitted (Fig 2). AHN-1 did not immunoprecipitate any radiolabeled proteins from extracts of resting neutrophils labeled for 25 minutes at 0°C in the presence of Na^{125}I but not lactoperoxidase (Fig 2, lane A). In contrast, when neutrophils were allowed to phagocytose EA7S for 30 minutes at 37°C and were cooled to 0°C and then labeled as above with Na^{125}I without lactoperoxidase, AHN-1 did immunoprecipitate radiolabeled proteins (Fig 2, lane C), although the quantity of ^{125}I -labeled proteins immunoprecipitated was much less than that observed when the radiolabeling mixture contained lactoperoxidase. Indeed, it was necessary to overexpose lanes B and E of the autoradiograph markedly to demonstrate the autoiodination shown in lanes C and D. However, the electrophoretic patterns of ^{125}I -labeled proteins in AHN-1 immunoprecipitates from cells radiolabeled after phagocytosis of EA7S was similar

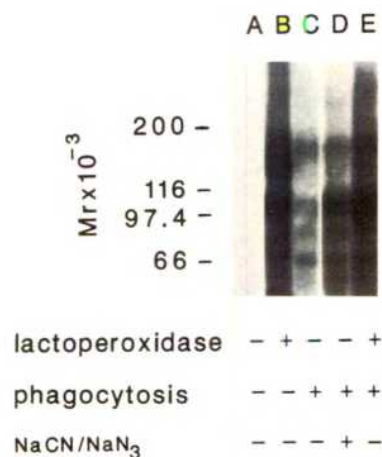


Fig 2. Immunoprecipitation with AHN-1 of neutrophil proteins radiolabeled with ^{125}I with and without lactoperoxidase. Neutrophils were labeled with ^{125}I for 25 minutes at 0°C as described in the text, except that for some reactions lactoperoxidase was omitted and in others NaCN and NaN_3 were added as indicated. Radiolabeled proteins were immunoprecipitated with monoclonal antibody AHN-1 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 5% to 15% linear gradient polyacrylamide gel and autoradiography as described in the text. Lanes A and B: Resting cells labeled without (lane A) or with (lane B) lactoperoxidase. Cells were also allowed to phagocytose EA7S without (lane C) and with (lane D) 1 mmol/L of NaCN and 1 mmol/L of NaN_3 and then were washed once in phosphate-buffered saline (PBS) at 0°C and labeled with ^{125}I without lactoperoxidase. Lane E: cells labeled with lactoperoxidase after phagocytosis of EA7S. Immunoprecipitation reactions in lanes B and E each contained 1×10^6 cell equivalents, whereas those in lanes A, C, and D each contained 2.5×10^6 cell equivalents. When 1×10^6 cell equivalents were used for the immunoprecipitation reactions in lanes A, C, and D, less radiolabeled protein was visualized. Lane B corresponds to Fig 1, lane B. Lane E corresponds to Fig 1, lane F. The autoradiograph was deliberately overexposed to demonstrate the autoradiolabeling in lanes C and D. Mol-wt standards were the same as in Fig 1. A duplicate experiment gave similar results.

whether lactoperoxidase was present (Fig 1, lane F) or absent (Fig 2, lane C). Similar studies of rabbit peritoneal polymorphonuclear leukocytes showed that 1 mmol/L of NaCN inhibited autoiodination during phagocytosis.⁶ In the present study, however, the addition of 1 mmol/L of NaCN and 1 mmol/L of NaN_3 did not eliminate autoiodination of the proteins recognized by AHN-1 (Fig 2, lane D).

Changes in surface proteins during phagocytosis detected by ^3H labeling. Changes in neutrophil surface glycoproteins occurring during phagocytosis were also examined by NaB^3H_4 labeling using three different techniques. Various proteins were radiolabeled by NaB^3H_4 following pretreatment with neuraminidase and galactose oxidase (NA + GO) (Fig 3, lane B), galactose oxidase alone (GO) (Fig 3, lane C), or NaIO_4 (Fig 3, lane D), but none was labeled without pretreatment (Fig 3, lane A). In contrast to the autoradiolabeling observed with ^{125}I labeling, the addi-

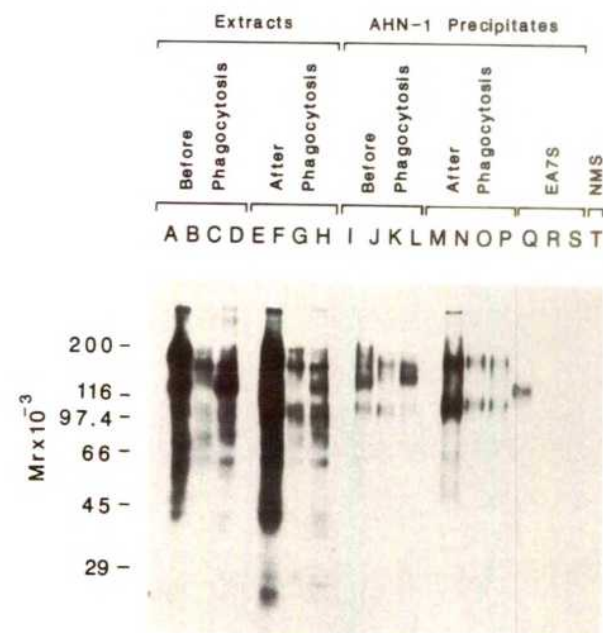


Fig 3. Surface glycoproteins of neutrophils labeled with ^3H before or after phagocytosis. Neutrophils were labeled with ^3H before (lanes A through D, I through L, and T) or after (lanes E through H, and M through P) phagocytosis of EA7S, solubilized, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 5% to 15% gradient polyacrylamide gel and autoradiography as described in the text. Lanes A through H: Cell extracts ($\sim 10^6$ cell equivalents per lane). Lanes I through T: Immunoprecipitates ($\sim 8 \times 10^6$ neutrophil cell equivalents per immunoprecipitation reaction in lanes I through P and T, or $\sim 8 \times 10^6$ RBC equivalents in lanes Q through S). Lanes I through S: Immunoprecipitates using AHN-1. Lane T: Immunoprecipitate using NMS. Cells were labeled with NaB^3H_4 following treatment with NA + GO (lanes B, F, J, N, and T), GO (lanes C, G, K, and O), NaIO_4 (lanes D, H, L, and P), or no pretreatment (lanes A, E, I, and M). EA7S target cells were also immunoprecipitated with AHN-1 after radiolabeling with ^3H using NA + GO (lane Q), GO (lane R), and NaIO_4 (lane S). Immunoprecipitates from extracts of cells labeled using GO or NaIO_4 using NMS resembled that shown in lane T. Mol-wt standards were the same as in Fig 1 with the addition of carbonic anhydrase, 29,000. A duplicate experiment gave similar results.

tion of NaB^3H_4 to neutrophils before or after phagocytosis, without pretreatment with $\text{NA} + \text{GO}$, GO , or NaIO_4 , did not result in radiolabeling of any proteins (Fig 3, lanes A and E, and data not shown). The electrophoretic patterns of ^3H -labeled proteins were similar to those previously reported.²³ Some changes in the proteins radiolabeled occurred when cells were labeled by these same techniques after phagocytosis of EA7S (Fig 3, lanes E through H). Several of these ^3H -labeled proteins were specifically immunoprecipitated by AHN-1 (Fig 3, lanes I through P) but not by normal mouse serum (Fig 3, lane T). AHN-1 immunoprecipitated much less ^3H -labeled protein in the mol-wt range of $\sim 140,000$ from extracts of cells labeled after phagocytosis of EA7S (Fig 3, arrow), similar to that observed with ^{125}I surface labeling. These changes could not be attributed to immunoprecipitation of radiolabeled target proteins (Fig 3, lanes Q through S). Although a small amount of radiolabeled protein was immunoprecipitated from EA7S labeled with the $\text{NA} + \text{GO}-\text{NaB}^3\text{H}_4$ technique by both AHN-1 (Fig 3, lane Q) and NMS (not shown), relatively little or no ^3H -labeled protein of this mol wt was immunoprecipitated by AHN-1 from neutrophils labeled by the same technique after phagocytosis of EA7S (Fig 3, lane N). Immunoprecipitates using AHN-1.1 were identical to those using AHN-1 (data not shown).

Because treatment with $\text{NA} + \text{GO}$ and NaB^3H_4 was the most effective method of labeling neutrophil surface glycoproteins recognized by AHN-1 with ^3H , this technique was used to label cells with ^3H before and after phagocytosis of the same target particles studied with ^{125}I labeling. As with ^{125}I labeling, the patterns of radiolabeled proteins immunoprecipitated by AHN-1 from cells labeled with ^3H prior to phagocytosis of EA7S, EAC423, or latex particles were similar to that obtained from cells labeled and solubilized without phagocytosis (data not shown). Immunoprecipitates with AHN-1 of cells labeled after phagocytosis of EA7S (Fig 4, lane C) or EAC423 (Fig 4, lane D) contained markedly less of the ~ 140 kD ^3H -labeled protein (Fig 4, arrow) than did cells labeled before phagocytosis (Fig 4, lanes A and B). As with ^{125}I labeling, phagocytosis of latex particles (Fig 4, lane E) or E (data not shown) before radiolabeling had little effect on the radiolabeled proteins immunoprecipitated by AHN-1. Normal mouse serum did not immunoprecipitate radiolabeled proteins from any of the samples (data not shown). Immunoprecipitates using AHN-1.1 were identical to those using AHN-1.

Analysis of surface protein transport into phagocytic vesicles by ^{125}I radiolabeling. During phagocytosis, the ingested particle is enveloped by a vesicle derived from the plasma membrane. To define the changes in neutrophil surface proteins during phagocytosis further, we isolated phagocytic vesicles from neutrophils using the technique of Stossel and colleagues.²⁶⁻²⁸ When phagocytic vesicles were prepared from neutrophils labeled at the cell surface with ^{125}I before phagocytosis of ORO particles, most of the major ^{125}I -labeled proteins found on the resting cell (Fig 5, lane A) were found in the phagocytic vesicles (Fig 5, lane B). Many of the ^{125}I -labeled proteins present in these phagocytic vesicles were specifically immunoprecipitated by AHN-1 (Fig 5,

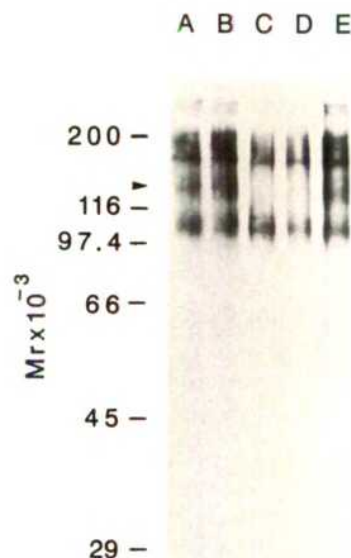


Fig 4. Immunoprecipitation by AHN-1 of radiolabeled surface glycoproteins of neutrophils labeled with ^3H before or after phagocytosis of various target particles. Neutrophils were labeled with ^3H using $\text{NA} + \text{GO}$ before or after phagocytosis of various target particles, solubilized, immunoprecipitated, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% polyacrylamide gel, and visualized by autoradiography as described in the text. A 7.5% gel was used for better resolution of the higher mol-wt proteins immunoprecipitated by AHN-1. Lane A: Resting cells. Lane B: Cells labeled after incubation in Hanks' balanced salt solution (HBSS) under conditions of phagocytosis but in the absence of target particles. Lanes C through E: cells labeled after phagocytosis of EA7S (lane C), EAC423 (lane D), or latex particles (lane E). Mol-wt standards were the same as in Fig 3. A duplicate experiment gave similar results.

lane C) but not by normal mouse serum (Fig 5, lane D). Immunoprecipitates from the plasma membrane fraction were similar but contained less radiolabeled material (not shown). In contrast, phagocytic vesicles prepared from neutrophils that phagocytosed ORO particles before radiolabeling contained little radiolabeled material (Fig 5, lane E) and no radiolabeled proteins were immunoprecipitated by AHN-1 or NMS (Fig 5, lanes F and G). As expected, immunoprecipitates using AHN-1.1 were identical to those using AHN-1 (not shown). Similar results occurred when phagocytic vesicles were prepared using nonopsonized ORO (data not shown).

Analysis of surface protein transport into phagocytic vesicles by ^3H radiolabeling. Transport of surface proteins into phagocytic vesicles was also analyzed after radiolabeling with ^3H using $\text{NA} + \text{GO}$. As with ^{125}I labeling, phagocytic vesicles prepared from neutrophils labeled with ^3H before phagocytosis of ORO particles (Fig 6, lane B) contained most of the major ^3H -labeled proteins present on resting cells (Fig 6, lane A). Phagocytic vesicles prepared from cells that were ^3H labeled after phagocytosis contained little ^3H -labeled protein (Fig 6, lane E). Many of the major ^3H -labeled proteins present in the phagocytic vesicles were specifically immunoprecipitated by AHN-1 (Fig 6, lane C) but not by normal mouse serum (Fig 6, lane D). Again,

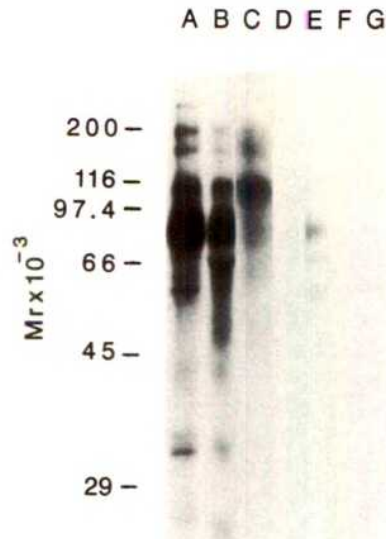


Fig 5. Isolation of phagocytic vesicles from neutrophils surface labeled with ^{125}I . Neutrophils were radiolabeled at the cell surface with ^{125}I using lactoperoxidase before or after phagocytosis of ORO particles. Phagocytic vesicles were then isolated, solubilized, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel and autoradiography as described in the text. Lane A: Surface-labeled resting neutrophils. Lanes B through D: ^{125}I -Labeling before phagocytosis. Lanes E through G: ^{125}I -Labeling after phagocytosis. Lanes B and E: Extracts of phagocytic vesicles. Extracts of phagocytic vesicles were immunoprecipitated with AHN-1 (lanes C and F) or normal mouse serum (lanes D and G). Equivalent volumes of the phagocytic vesicle fractions were added to lanes B and E and to the immunoprecipitation reactions for lanes C, D, F, and G. Mol-wt standards were the same as in Fig 3. A duplicate experiment gave similar results.

immunoprecipitates using AHN-1.1 were identical to those using AHN-1 (not shown). Similar results occurred when phagocytic vesicles were prepared using nonopsonized ORO (data not shown).

DISCUSSION

Phagocytosis is an important defense mechanism of granulocytes and mononuclear phagocytes. The process of phagocytosis involves particle recognition, binding, and ingestion. During phagocytosis, the plasma membrane of the phagocyte gradually envelops the surface of the target particle and then the invaginated membrane fuses, separating from the plasma membrane to create a phagocytic vesicle. These vesicles then fuse with lysosomes to create phagolysosomes. The membrane of the phagocytic vesicle is clearly derived from the phagocyte's plasma membrane, but the composition of neither membrane is well defined. Glycoproteins of the plasma membrane are important in cell function, and several surface glycoproteins are presumably involved in phagocytosis. A better understanding of the mechanism of phagocytosis will require more detailed knowledge of the composition of the plasma membrane and the phagocytic vesicle and the changes in membrane composition that occur during phagocytosis.

The fates of human neutrophil surface glycoproteins dur-

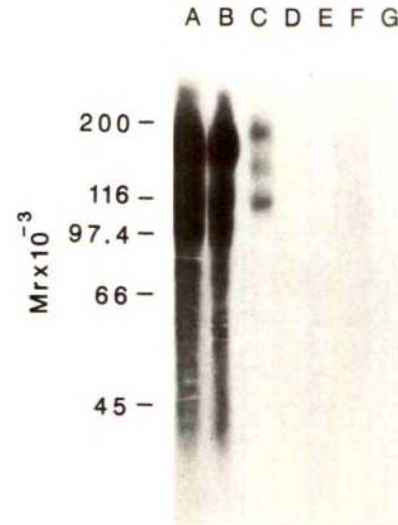


Fig 6. Isolation of phagocytic vesicles from neutrophils surface labeled with ^3H . Neutrophils were radiolabeled with ^3H before or after phagocytosis of ORO particles using NaB^3H_4 after treatment with NA + GO as described in the text. Phagocytic vesicles were then isolated, solubilized, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% polyacrylamide gel and autoradiography as described in the text. Lane A: Surface-labeled resting neutrophils. Lanes B through D: ^3H Labeling before phagocytosis. Lanes E through G: ^3H -labeling after phagocytosis. Lanes B and E: Extracts of phagocytic vesicles. Extracts of phagocytic vesicles were immunoprecipitated with AHN-1 (lanes C and F) or normal mouse serum (lanes D and G). Equivalent volumes of the phagocytic vesicle fractions were added to lanes B and E and to the immunoprecipitation reactions for lanes C, D, F, and G. Mol-wt standards were the same as in Fig 1. A duplicate experiment gave similar results.

ing phagocytosis are not well defined. Studies of phagocytic vesicles of cultured murine peritoneal macrophages, using a technique of intralysosomal iodination, have suggested that following antibody- and complement-independent phagocytosis of latex beads, the membrane proteins accessible to lactoperoxidase-catalyzed iodination on the luminal surface of the phagolysosome and on the external face of the plasma membrane are similar.¹⁰ A companion study provided evidence for continuous bidirectional flow of membrane polypeptides between the plasma membrane and the secondary lysosome.¹¹ Studies of both rabbit polymorphonuclear leukocytes and alveolar macrophages showed that the activities of five separate specific transport systems were unaffected by phagocytosis, even after 35% to 50% of the surface membrane had been internalized.⁵ This work supported the concept that the membrane is mosaic in character, with geographically separate transport and phagocytic sites, and that during phagocytosis, transport sites are preserved on the surface despite the internalization of a large amount of the plasma membrane.⁵ Further studies suggested that colchicine-sensitive sites were involved in this differential internalization, since colchicine treatment resulted in a decrease in surface transport sites during phagocytosis.⁷ Other workers studying human neutrophils found a decreased but differentially reduced activity of transport sites for potassium and lysine following phagocytosis of zymosan or latex, also

favoring a mosaic membrane structure.⁸ Other studies of antibody-dependent phagocytosis of lipid vesicles by RAW264 macrophages found a loss of surface Fc-receptor activity with no loss of C3b-receptor activity, again demonstrating selectivity in surface protein internalization during phagocytosis.¹² These same workers observed a loss of both Fc- and C3b-receptor activity from the cell surface following the uptake of latex beads, a comparatively nonspecific phagocytic process. Similar findings were observed in studies of murine macrophages.^{13,14} In addition, using the techniques of ¹²⁵I surface labeling and two-dimensional gel electrophoresis,⁹ an ~140-kd protein was observed to disappear from the surface of RAW264 macrophages following phagocytosis of opsonized liposomes, providing further evidence that phagocytosis leads to a modification of cell-surface protein composition.

Willinger and associates⁶ used lactoperoxidase-catalyzed iodination and phagocytosis of ORO to study the fate of surface proteins of rabbit polymorphonuclear leukocytes during phagocytosis. They found a nonrandom interiorization of ¹²⁵I-labeled surface proteins during phagocytosis with a reduction in the amount of three proteins of mol wt ~100,000, 180,000, and 215,000 on the cell surface after phagocytosis. These same proteins also appeared to be translocated to the phagosome during phagocytosis. The 100- and 180-kd proteins transported to the phagosome during phagocytosis by rabbit neutrophils may correspond to the 105-kd and 135- to 190-kd proteins recognized by AHN-1 in human neutrophils. Two similar studies on the L cell, a murine fibroblast, also analyzed the fate of external membrane proteins during phagocytosis.^{22,32} Both studies found all the proteins identified by ¹²⁵I surface labeling in isolated phagosomes following phagocytosis of latex beads when cells were grown in suspension. L cells grown in monolayer cultures contained only two major surface-iodinated proteins, one of which was found in phagosomes.³²

We used two techniques of cell-surface labeling to study changes in the surface protein composition of human neutrophils during phagocytosis. Such studies with ¹²⁵I surface labeling have been difficult because: (a) When stimulated, neutrophils can iodinate cellular proteins in the absence of added lactoperoxidase by their intrinsic halogenating systems³¹; (b) analysis by SDS-PAGE of human neutrophils surface labeled with ¹²⁵I using lactoperoxidase reveals a complex pattern of radiolabeled proteins; and (c) ¹²⁵I labeling after phagocytosis results in ¹²⁵I-labeled proteins derived from the target particle, further complicating analysis. We used immunoprecipitation and cell-surface labeling with both ¹²⁵I using lactoperoxidase and also ³H using NA + GO, GO, or NaIO₄, to study changes in the surface protein composition of human neutrophils during phagocytosis. Protein radiolabeling with ³H was only observed following exogenous enzyme or NaIO₄ treatment, even in stimulated neutrophils. Because the electrophoretic pattern of radiolabeled proteins obtained from surface-labeled neutrophils was complex, we used immunoprecipitation with the granulocyte-specific monoclonal antibody of AHN-1 to restrict our analysis to a subset of surface proteins. AHN-1 binds an oligosaccharide epitope present on several surface glycopro-

teins of human neutrophils¹⁷ but it had no reactivity with any of the target particles, thus eliminating interference with radiolabeled target proteins. Analysis of the surface proteins recognized by AHN-1 is of particular interest because AHN-1 inhibits phagocytosis by human neutrophils,¹⁸ suggesting that one or more of these proteins is intimately involved in the process of phagocytosis.

We found that AHN-1 immunoprecipitated less of certain ¹²⁵I-labeled proteins when neutrophils were surface labeled after phagocytosis. In addition, there appeared to be a selective decrease in two ¹²⁵I-labeled proteins of ~140 kd and 170 kd after phagocytosis of IgG or C3 opsonized targets but not after phagocytosis of latex particles or ORO. These results were confirmed by a similar analysis of cells labeled at the surface with NaB³H₄ using three different labeling techniques. Similar studies using the CDw18 monoclonal antibody 60.3 revealed that 60.3 also immunoprecipitated less of the ¹²⁵I-labeled α and β chains of this protein family from cells surface labeled after phagocytosis (data not shown). Other studies have reported that phagocytosis results in an increase in surface CR3 recognized by the Mol monoclonal antibody, due to translocation to the surface from intracellular pools.^{33,34} The decrease we observed in the quantity of ¹²⁵I-labeled proteins immunoprecipitated by 60.3 from cells labeled after phagocytosis could be due to less efficient radiolabeling due to the presence of the target particles (for example, steric hindrance or receptor occupancy).

The changes we observed in the proteins immunoprecipitated by AHN-1 may have been due to internalization of proteins resulting in fewer of these proteins exposed on the cell surface, binding of target particles resulting in less efficient radiolabeling, or some other conformational change resulting in less efficient labeling of the proteins under study. To define the fate of these proteins during phagocytosis further, we isolated phagocytic vesicles from neutrophils surface labeled before or after phagocytosis of ORO particles, using the technique of Stossel and colleagues.²⁶⁻²⁹ These studies, using either ¹²⁵I or ³H labeling, demonstrated that three proteins of ~105 kd, 140 kd, and 170 kd recognized by AHN-1 were internalized in the phagocytic vesicle. Similar studies revealed that the β chain and all three α chains recognized by the CDw18 monoclonal antibody 60.3 were also internalized into ORO containing phagocytic vesicles (data not shown). The apparently selective internalization of the 140-kd and 170-kd proteins recognized by AHN-1 compared with the 105-kd protein during phagocytosis of EA7S or EAC423 could be due to several possibilities: (a) The 140-kd and 170-kd proteins were internalized at a greater rate than was the 105-kd protein; (b) all three proteins were internalized at the same rate, but resting cells contain less of the 140-kd and 170-kd proteins on the surface than of the 105-kd protein; (c) the target particles bound more closely to the 140-kd and 170-kd proteins, making them less accessible to radiolabeling; or (d) some other conformational change during particle binding and internalization altered the labeling efficiency of the proteins. We did not observe more internalization of the ~140-kd protein into the phagocytic vesicle than of the 105-kd or 170-kd proteins

during phagocytosis of ORO. Similarly, the relative amounts of ^{125}I -labeled 105-kd, 140-kd, and 170-kd proteins recognized by AHN-1 remaining in the plasma membrane fraction of cells labeled at the surface before phagocytosis of ORO was similar to that observed in resting cells. Thus, our results with phagocytosis of ORO do not provide evidence for selective internalization of the 140-kd protein in comparison to the 105-kd and 170-kd proteins or for the presence of a greater number of 105-kd and 170-kd proteins than 140-kd proteins on the surface of resting cells coupled with equal rates of internalization. However, the whole cell immunoprecipitation studies described in Fig 1 did not reveal a selective decrease in the quantity of ^{125}I -labeled 140-kd protein immunoprecipitated by AHN-1 from cells labeled after phagocytosis of ORO. The selective decrease in radiolabeled 140-kd and 170-kd proteins immunoprecipitated by AHN-1 from cells labeled at the surface after phagocytosis of EA7S or EAC423 compared with resting cells may be due to a selective decrease in labeling efficiency of these proteins resulting from target binding or a subsequent step in phagocytosis.

In summary, this report demonstrates that qualitative changes in the surface membrane composition of human neutrophils occur during phagocytosis. Some of the molecules that take part in this alteration have been identified using the granulocyte-specific monoclonal antibody AHN-1. Similar studies using other monoclonal antibodies that react with the phagocyte surface may be informative. These results confirm that a nonrandom internalization of surface proteins occurs during phagocytosis by human neutrophils, as has been observed in studies of some other cell systems^{5-9,12-14,32} but not in others.^{10,22,32} Such nonrandom internalization of surface proteins may be important in preserving other independent membrane functions during phagocytosis.

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Immunological Specificity and Mechanism of Action of IgG Lupus Anticoagulants

By Vittorio Pengo, Perumal Thiagarajan, Sandor S. Shapiro, and Marilyn J. Heine

Although observations have implied that lupus anticoagulants have immunologic specificity toward anionic phospholipids, this assumption has been directly demonstrated in only one patient with a monoclonal IgM paraprotein. We tested the generality of this hypothesis directly by isolating five IgG lupus anticoagulants from patients with lupuslike syndromes and/or thrombosis. IgG lupus anticoagulant fractions were isolated free of other plasma proteins and free of contaminating phospholipid by adsorption to and elution from cardiolipin-cholesterol-dicetyl phosphate liposomes, followed by chromatography on protein A-Sepharose. Cardiolipin liposomes, but not phosphatidylcholine liposomes, were capable of removing all, or nearly all, lupus anticoagulant activity from patient plasma. The affinity-purified IgG preparations reacted with cardiolipin,

phosphatidylserine, phosphatidylinositol, and phosphatidic acid, but not with phosphatidylcholine or phosphatidylethanolamine, and inhibited calcium-dependent binding of prothrombin and of factor X to phosphatidylserine-coated and to cardiolipin-coated surfaces. F(ab')₂ fragments retained lupus anticoagulant activity and bound to cardiolipin in an enzyme-linked immunosorbent assay (ELISA). Anticardiolipin and lupus anticoagulant activity were both present in acidic fractions on isoelectric focusing. These data strongly suggest that most, if not all, lupus anticoagulants are antibodies that have immunologic specificity towards anionic phospholipids, thereby blocking the calcium-mediated binding of vitamin K-dependent coagulation factors to coagulation-active phospholipid surfaces.

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LUPUS ANTICOAGULANTS are acquired inhibitors of coagulation tests that occur in systemic lupus erythematosus (SLE), in various other clinical settings, and in apparently normal persons.¹

Because the lupus anticoagulant effect is noted in all phospholipid-dependent coagulation tests²⁻⁶ and because of the correlation between the presence of lupus anticoagulants and reactivity against cardiolipin in solid-phase immunoassays,⁷⁻¹¹ lupus anticoagulants have been widely assumed to be antibodies reactive against anionic phospholipids. Actually, the immunologic nature and mechanism of action of the lupus anticoagulant effect have only been demonstrated in one patient.¹² In 1980, our laboratory¹² clarified the mechanism of action of an IgM λ paraprotein with lupus anticoagulant activity. The purified paraprotein showed immunologic reactivity against the anionic phospholipids phosphatidylserine, phosphatidylinositol, and phosphatidic acid, but did not react with the neutral phospholipids phosphatidylcholine or phosphatidylethanolamine. This protein inhibited the binding of prothrombin and factor X to phospholipid micelles, thereby accounting for the prolongation of phospholipid-dependent coagulation tests.

Since this initial work, it has been widely assumed, although not rigorously demonstrated, that all lupus anticoagulant activity is due to the same mechanism. However, other authors have stressed the heterogeneity of the lupus anticoagulant patient population, as well as the apparent variability in the response of lupus anticoagulant tests to the presence of platelets,¹³ to argue against a common mechanism of action. Interestingly, neither the original patient, nor four other IgM lupus anticoagulants we subsequently studied, nor IgM lupus anticoagulants reported by others,^{14,15} have been associated with thrombosis. Most lupus anticoagulants, on the other hand, occur in a setting of polyclonal increases in immunoglobulins and are associated with a high incidence of thrombosis.^{1,16-21} To assess whether there is indeed a general mechanism for the lupus anticoagulant effect, a technique for isolating lupus anticoagulants from plasma of patients without monoclonal paraproteins is necessary. The preparation must be a pure immunoglobulin free of contaminating phospholipid. We developed a technique that

uses affinity purification on cardiolipin liposomes followed by protein A-Sepharose chromatography to prepare phospholipid-free purified lupus anticoagulants. We isolated five IgG lupus anticoagulants, four from patients with a history of thrombosis, and examined the immunologic nature of their interaction with phospholipids and the mechanism of their inhibition of phospholipid-dependent coagulation tests.

MATERIALS AND METHODS

Human thrombin was a gift from Dr John Fenton, New York State Department of Health Laboratories, Albany. Pepsin was purchased from Worthington Biochemical, Freehold, NJ. Protein A-Sepharose, Sephacryl G-75 (Superfine) and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Ampholines were purchased from LKB, Bromma, Sweden. Phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, phosphatidic acid, and phosphatidylcholine were obtained from Supelco, Bellefonte, PA. Cardiolipin, dicetyl phosphate, cholesterol, alkaline phosphatase-labeled goat antibodies to human IgG, human IgM, and rabbit IgG, as well as *p*-nitrophenyl phosphate and bovine serum albumin (BSA) (fraction V) were purchased from Sigma Chemical, St Louis. The purity of phospholipids was checked by thin-layer chromatography.²² Russell viper venom was obtained from Burroughs Wellcome, Raleigh, NC, and Thrombofax was purchased from Ortho Diagnostic Systems, Raritan, NJ. Antisera to human IgG, IgA, and IgM were obtained from Meloy Laboratories,

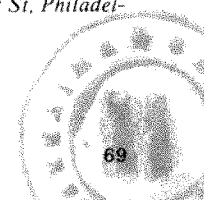
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Springfield, VA. Standard laboratory chemicals were supplied by Fisher Scientific, Fair Lawn, NJ. Carrier-free ^{125}I was obtained from Cambridge Nuclear, Billerica, MA.

IgG Purification

Lupus anticoagulant plasma was clotted by incubation with 2 U/mL of human thrombin for 2 hours at 37°. Fibrin was removed by centrifugation at 4°C for 10 minutes at 14,000 g. γ -Globulins were precipitated from the supernatant serum by addition at 4°C of an equal volume of saturated ammonium sulphate. After 25 minutes of stirring, the precipitate was recovered by centrifugation in the cold for 10 minutes at 14,000 g. The precipitate was dissolved in approximately one-seventh the original plasma volume, using Tris-buffered saline (TBS: 0.02 mol/L of Tris, 0.15 mol/L of NaCl, pH 7.4), and applied to a 2.5×90 -cm column of Sephacryl S-300 equilibrated with TBS. Fractions of 2.5 mL were collected and tested qualitatively for IgG, IgM, and IgA by Ouchterlony double diffusion and for lupus anticoagulant activity by their ability to prolong the dilute Russell viper venom time (RVVT) of normal plasma.^{23,24} Fractions containing lupus anticoagulant activity were pooled and applied to a 1×5 -cm column of protein A-Sepharose equilibrated with TBS. The lupus anticoagulant activity, in every case, was retained on the column and was eluted using a buffer consisting of 0.1 mol/L of glycine, 0.5 mol/L of NaCl, pH 3, and immediately neutralized with 1 mol/L of Tris buffer, pH 8.4. Eluates were dialyzed against TBS before being tested for lupus anticoagulant activity. Control IgG was prepared from normal plasma in an identical manner. IgG prepared in this manner is free of other immunoglobulins and shows no bands other than IgG on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after staining with Coomassie blue.

F(ab')₂ Preparation

After dialysis against acetate buffer (0.2 mol/L of Na acetate, 0.2 mol/L of NaCl, pH 4), purified IgG (2 to 3 mg/mL) was incubated at 37°C for 20 hours with freshly prepared pepsin at a weight ratio of 50:1.²⁵ At the end of the 20-hour incubation, no intact IgG was detectable by SDS-PAGE. The reaction was stopped by adding solid Tris (5 to 10 mg). The digested material was dialyzed against TBS and applied to a 1×5 -cm protein A-Sepharose column. The flowthrough, containing the F(ab')₂ fragments, was concentrated by Amicon ultrafiltration using PM-10 membranes (Amicon, Lexington, MA) and tested for lupus anticoagulant and anticardiolipin activities. The protein A-Sepharose flowthrough was free of whole IgG and Fc-fragments, as judged by SDS-PAGE and by double diffusion against antiserum to human IgG (Fc-specific).

ELISA for Anticardiolipin Antibodies

One microgram of cardiolipin in 25 μL of ethanol was added to each well of a 96-well microtiter plate (Falcon #3911, Becton Dickinson, Oxnard, CA) and evaporated under a stream of nitrogen for 20 minutes, after which the wells were washed twice with 100 μL of phosphate-buffered saline (PBS: 8 g/L of NaCl, 0.2 g/L of KH_2PO_4 , 2.9 g/L of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g/L of KCl, pH 7.4). All subsequent steps were carried out at room temperature. One hundred microliters of 10% fetal calf serum (FCS) in PBS was then added to each well and incubated for 2 hours to block nonspecific binding. After the plates were washed six times with PBS, 100 μL of plasma diluted in PBS containing 10% FCS was added to each well and incubated for 1 hour. The wells were washed six times with PBS, after which 100 μL of a 1:1000 dilution of alkaline phosphatase-labeled goat anti-human IgG or goat anti-human IgM was added and incubated for 1 hour. The plates were then washed six times more in PBS, and 100 μL of freshly prepared *p*-nitrophenyl phos-

phate solution (1 mg/mL in 10% diethanolamine buffer, pH 9.8) was added to each well. After 1-hour incubation, the absorbance was measured at 405 nm, using a Titertek Multiscan plate reader (Flow Laboratories, McLean, VA). In experiments using F(ab')₂, 100 μL of a 1:400 dilution of rabbit anti-human F(ab')₂ (Jackson Laboratories, Avondale, PA) was used as a second antibody, and alkaline phosphatase-labeled goat anti-rabbit IgG was used as the developing antibody.

The ELISA assay for antibody activity against other phospholipids was performed in a similar manner, except that: (a) Two micrograms of phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylethanolamine, or phosphatidylcholine in 10 μL of chloroform was added to each well of a 96-well microtiter plate containing 30 μL of ethanol; (b) when phospholipid reactivity of eluates from cardiolipin liposomes was studied, 100 μL -aliquots of eluate were used; and (c) the reaction was stopped after 15 minutes.

Isoelectric Focusing

Forty grams of Sephadex G-75 was washed and prepared as described by the manufacturer, and finally suspended in 90 mL of deionized water. To the Sephadex was added: 2.5 mL of Ampholine, pH 5 to 7; 2.5 mL of Ampholine, pH 7 to 9; and 5 mL of purified patient IgG (10 mg/mL in 1% glycine). After gentle stirring, the mixture was poured into a 10×20 -cm glass tray, and excess water was evaporated under a stream of air. Focusing was performed at 10° for 15 hours at 8 W constant power. The gel was then cut into six fractions. A small amount of each fraction was resuspended in deionized water for pH measurement; the remainder of each fraction was resuspended in 5 mL of TBS in a small column, and the proteins were eluted by washing the column with 5 mL of TBS. Column eluates were dialyzed against TBS and tested for lupus anticoagulant and anticardiolipin activities.

Analytical isoelectric focusing was performed in an LKB Multiphor apparatus on 5% polyacrylamide gels containing 2.2% (wt/vol) Ampholine, pH 5.5 to 8.5. Purified IgG or liposome eluates were dialyzed against deionized water, applied to the gel, and run at 50 mA with a constant power of 25 W. The gels were fixed for 1 hour in a solution of 5% trichloroacetic acid and 5% sulfosalicylic acid and stained using the Bio-Rad silver-staining kit (Bio-Rad Laboratories, Richmond, CA).

Immunoaffinity Purification of Anticardiolipin Antibodies

Liposomes were prepared essentially according to the method of Kinsky.²⁶ A mixture of cardiolipin, cholesterol and dicetyl phosphate (molar ratio 10:15:2) in chloroform was dried in a stream of nitrogen in a 25-mL glass tube. The lipids were resuspended in 1 mL of 0.15 mol/L of NaCl by vigorous agitation, using a vortex mixer. After resuspension, the concentration of phospholipids was 5.3 to 5.9 mg/mL. Ten milliliters each of saline and plasma were added to 1 mL of liposome suspension, and the mixture was incubated at 4°C for 30 minutes. After centrifugation at 27,000 g for 10 minutes at 4°C, the precipitate was washed three times with 0.15 mol/L of NaCl. The precipitate was resuspended by vigorous agitation in 2 mL of 3 mol/L of NaCl, following which the suspension was added to a separatory funnel containing 10 mL of ethyl ether.²⁷ After settling, the clear aqueous layer was removed from the bottom of the funnel, centrifuged, and dialyzed in the cold against three changes of TBS. The dialyzed eluate was tested for lupus anticoagulant activity by the dilute RVVT, for immunoglobulin class by Ouchterlony double diffusion, and for reactivity against phospholipids by enzyme-linked immunosorbent assay (ELISA). The eluate was further purified by protein A-Sepharose column chromatography, as previously described, and retested for purity and for biological activities.

Labeling of Cardiolipin with ^{125}I

Cardiolipin was radioiodinated directly by the chloramine-T method, as described by Antonov and colleagues.²⁸ Thin-layer chromatography demonstrated that ~60% of the added radioactivity had been incorporated into cardiolipin. Most of the remainder traveled with the solvent front. Labeled cardiolipin had the same mobility as standard cardiolipin. The spot containing the radioactive cardiolipin was extracted from the silica gel plate as described previously.²² When re-run on thin-layer chromatography, this extracted labeled material showed a single spot with the mobility of standard cardiolipin; no radioactivity was present in the solvent front. On the basis of phosphorus content,²⁹ the specific radioactivity of ^{125}I -cardiolipin was calculated as 26 mCi/mmol.

Coagulation Studies and Assessment of Lupus Anticoagulant Activity

Venous blood was collected in one-tenth volume of 3.8% trisodium citrate, and plasma was obtained by centrifugation in the cold at 2,300 g for 15 minutes. Routine coagulation studies were performed as previously described.¹² Lupus anticoagulant activity of patient plasma was assessed by the dilute RVVT, as previously described.^{23,24} In our laboratory, the dilute RVVT of normal plasma is 25.6 ± 2.6 seconds (± 2 SD). Patients were not considered to have lupus anticoagulants unless their dilute RVVT was >30 seconds and was not corrected by addition of an equal volume of normal plasma. The anticoagulant activity of IgG or of F(ab')_2 was measured in the dilute RVVT by adding 200 μL of the dialyzed test material, instead of the usual 100 μL , to this system. Addition of 200 μL of TBS alone to normal plasma gives a dilute RVVT of 27 to 30 seconds.

Prothrombin and Factor X Binding to Phospholipid

Prothrombin was purified from normal plasma by a slight modification³⁰ of the method of Morrison and Esneuf,³¹ and labeled with ^{125}I by the Iodogen method³² to a specific activity of 231 Ci/mmol. Factor X was purified according to the method of Miletich and co-workers,³³ with some modifications described previously,¹² and labeled with ^{125}I by the Iodogen method to a specific activity of 60 Ci/mmol. Two micrograms of phospholipid was added to each well of a microtiter plate and dried and washed as described above. Two hundred microliters of TBS containing 1 mg/mL of BSA was then added, to block nonspecific binding, and the plates were incubated for 1 hour. After plates were washed once with TBS, 150 μL of TBS containing 1 mg/mL of BSA and 2 mmol/L of calcium was added, followed immediately by 50 μL of immunoaffinity-purified IgG (134 $\mu\text{g/mL}$) or control IgG (150 $\mu\text{g/mL}$) in TBS. The plates were incubated for 1 hour at room temperature, after which 10 μL of labeled prothrombin (1.4×10^6 cpm) or factor X (1.7×10^6 cpm) was added. After 10-minute incubation at room temperature, the wells were aspirated dry, and the bottoms of the wells were punched out and counted in an Isodyne γ -counter (Model 1185, G.D. Searle, Chicago). Maximum binding of prothrombin was determined by addition of TBS-albumin-calcium buffer alone, in the absence of any IgG. As a control, prothrombin and factor X binding were measured in the presence of 2 mmol/L of EDTA and were 3% and 11% of maximum binding, respectively. Residual binding, measured in the presence of a 50-fold excess of cold prothrombin or factor X, was subtracted from total binding, to give "specific" binding.

Patients

Patient no. 1. The patient was a 35-year-old man with a 10-year history of a manic-depressive illness for which he had been treated with lithium, chlorpromazine, and other tricyclic antidepressants. He had not received any medication except lithium in the 6 months

prior to study. He had had several episodes of deep vein thrombosis as well as at least one probable pulmonary embolism. He had a normal platelet count, and his serological test for syphilis (RPR) was negative. At the time of the study, he was receiving warfarin.

Patient no. 2. The patient was a 39-year-old woman with SLE of 8-year duration who had end-stage renal disease and was on hemodialysis. She had had a cerebrovascular accident at the age of 35, and also had a history of possible pulmonary emboli. She had occlusion of arteriovenous shunts in the forearm on at least two occasions. Her platelet count was in the low-normal range. She had a false-positive serological test for syphilis (RPR) at the time of diagnosis, but not at the time of study, when she was receiving 20 mg of prednisone every other day.

Patient no. 3. The patient was a 32-year-old woman with a history of three spontaneous abortions, after which she delivered a full-term live baby while on steroid therapy. She had a normal platelet count. She had had a false-positive serology (RPR) since her teens, but had no other signs of SLE.

Patient no. 4. The patient was a 49-year-old man with a 10-year history of SLE. He had an acute episode of right ileofemoral arterial occlusion 1 year earlier, at which time a lupus anticoagulant was discovered. He had a normal platelet count and a negative serological test for syphilis (RPR).

Patient no. 5. The patient was a 58-year-old woman with renal disease and possible SLE. She had no history of thromboembolic phenomena. She had a normal platelet count and a false-positive serology (RPR). At the time of the study, she was receiving 20 mg of prednisone daily.

As shown in Table 1, all five patients had a prolonged dilute RVVT, which did not normalize on addition of an equal volume of normal plasma. Four of the five patients had a prolonged activated partial thromboplastin time (APTT), whereas only one (patient no. 1, receiving warfarin) had a prolonged prothrombin time (PT).

RESULTS

Immunoglobulin Nature of the Lupus Anticoagulant and Anticardiolipin Activities

Lupus anticoagulant activity in all five patients gel-filtered on Sephacryl S-300 in the IgG peak. Three patients (patients no. 1, 3, and 5) had, in addition, a very small amount of inhibitory activity associated with the IgM peak. The maximum prolongation of the dilute RVVT in IgM fractions was 2 seconds, whereas the maximum prolongation in the IgG peak was 13 to 14 seconds. No prolongation of the RVVT was observed in any of the Sephacryl S-300 fractions of control plasma. The IgG peaks were further purified by chromatography on protein A-Sepharose. Glycine eluates of the protein A-Sepharose columns contained the inhibitory activity, whereas the flowthrough fractions had no lupus anticoagulant activity.

IgG anticardiolipin activity was detected in all five patient

Table 1. Screening Coagulation Tests

	Patient					Normal
	1	2	3	4	5	
APTT (s)	90.0	27.0	42.6	38.4	42.0	23-36
PT (s)	23.2	11.7	13.2	12.2	12.0	11.5-13.5
Dilute RVVT (s)						
Patient	38.2	33.4	34.0	36.5	46.3	23-28.2
Patient + normal (1:1)	36.6	30.4	29.9	32.0	42.7	23-28.2

plasmas; in addition, patients 1, 3, and 5 had weak IgM anticardiolipin activity.

F(ab')₂ fragments prepared from patient IgG prolonged the dilute RVVT of normal plasma in a dose-dependent manner; normal F(ab')₂ had only a minimal effect on this test (Fig 1A). Patient F(ab')₂ fragments also bound in a dose-dependent manner to cardiolipin in the ELISA, whereas control F(ab')₂ showed only minimal binding (Fig 1B).

Preparative isoelectric focusing of purified IgG from patient 1 (Fig 2) demonstrated a peak of lupus anticoagulant and anticardiolipin activity in the first fraction, pH 5.8; in contrast, IgG was distributed throughout all six fractions relatively equally.

Immunoaffinity Purification and Properties of Lupus Anticoagulant and Anticardiolipin Antibodies

As shown in Table 2, when IgG-containing fractions from a Sephacryl S-300 column (patient no. 5) were incubated with cardiolipin liposomes, nearly all lupus anticoagulant activity was adsorbed. When phosphatidylcholine liposomes were used instead, no lupus anticoagulant activity was

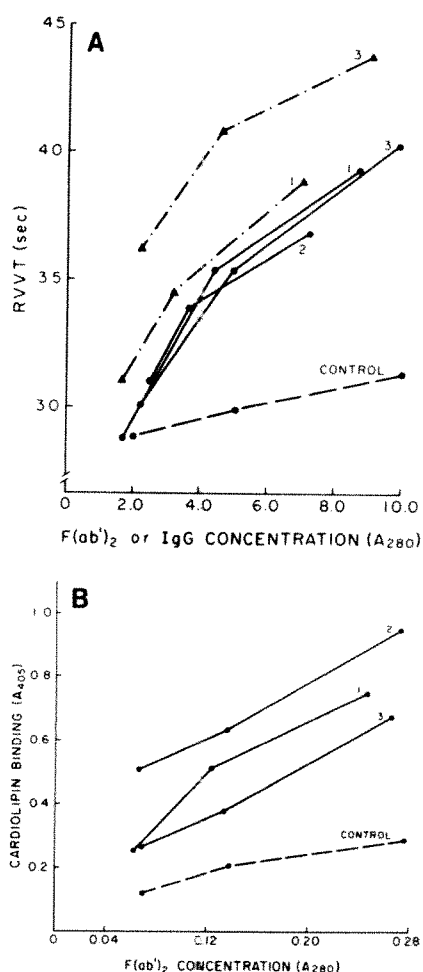


Fig 1. (A) Effect of F(ab')₂ fragments and whole IgG on the dilute RVVT of normal plasma. Control F(ab')₂, ●—●; patient F(ab')₂, ●—●; patient IgG, ▲—▲. Patients are identified by number. (B) Anticardiolipin activity of F(ab')₂ fragments.

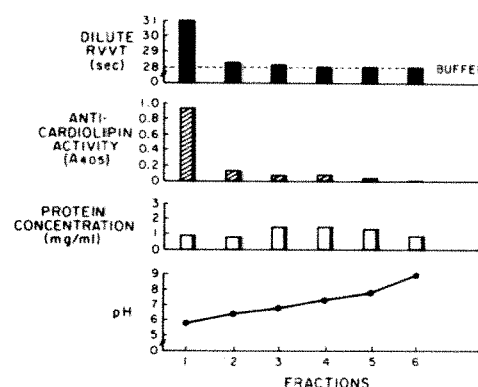


Fig 2. Preparative isoelectric focusing on Sephadex G-75 of IgG from patient no. 1, purified on protein A-Sepharose.

adsorbed. The cardiolipin liposome eluate contained lupus anticoagulant activity, whereas the phosphatidylcholine liposome eluate contained none.

Cardiolipin liposome eluates were prepared from patient and normal plasmas (Table 3). Eluates from patient plasma, in contrast to eluates from normal plasma, possessed lupus anticoagulant activity. When tested by Ouchterlony double diffusion, patient eluates contained IgG and albumin, whereas control eluates contained only albumin. Patient and control eluates were negative for IgM, IgA, and lipoproteins by the same method. Final purification of patient liposome eluates was achieved by chromatography on protein A-Sepharose. Eluates from this column were pure IgG preparations, as judged by double diffusion and by silver staining of SDS-PAGE gels. To estimate the cardiolipin contamination of the affinity-purified IgG, we purified one patient plasma with liposomes containing ¹²⁵I-cardiolipin. The starting liposome preparation contained 216,250 cpm. The centrifuged, dialyzed liposome eluate contained 219 cpm. After protein A-Sepharose chromatography, the eluted IgG contained only 42 cpm, representing <1 mol of cardiolipin/10¹⁰ mol of IgG.

The affinity-purified antibodies prolonged the dilute RVVT of normal plasma in a dose-dependent manner, as shown in Fig 3. A final concentration of as little as 3.3 μg/mL of this purified antibody produced a measurable increase in the clotting time. Control IgG had no effect. Based on the anticoagulant activity of the affinity-purified IgG, we estimate that lupus anticoagulants in these patients account for no more than 0.2% of the total plasma IgG.

Table 2. Specificity of Liposome Purification

Test Material	Dilute RVVT (s)
Sephacryl S-300 IgG pool	38.5
Liposome eluate*	
Cardiolipin	42.3
Phosphatidylcholine	29.9
Liposome supernatant*	
Cardiolipin	31.7
Phosphatidylcholine	41.1
Buffer	28.8

*After protein A-Sepharose chromatography.

Table 3. Lupus Anticoagulant Activity of Liposome Eluates From Patient and Normal Plasma

Patient	A ₂₈₀ Eluate	Dilute RVVT (Eluate/Buffer, s)
1	0.237	34.4/27.9
2	0.991	38.8/27.9
3	0.291	36.9/28.4
A	0.044	26.9/27.8
B	0.114	29.4/28.4

With the ELISA test system, the cardiolipin liposome eluates reacted with cardiolipin and, as shown for patient 1 in Fig 4, also reacted with phosphatidylserine, phosphatidylinositol, and phosphatidic acid, but not with phosphatidylethanolamine, phosphatidylcholine, or dicetyl phosphate. Identical results were obtained in all four patients tested (patients no. 1, 2, 3, and 5). Normal IgG, at approximately the same concentration as in patient eluates, showed no reactivity against any phospholipids. In addition, liposome eluates from normal plasma contained only trace amounts of protein and showed little or no reactivity.

Analytical isoelectric focusing of affinity-purified, phospholipid-free IgG from patients no. 2 and 3 (Fig 5) showed several bands of acidic pI; in contrast, the bulk of normal IgG focused in the alkaline range.

Inhibition of Prothrombin and Factor X Binding to Phosphatidylserine-coated Surfaces

The calcium ion-dependent binding of ¹²⁵I-prothrombin to phospholipid-coated surfaces was studied in microtiter plates. As expected, prothrombin bound to phosphatidylserine-coated wells (136 to 205 fmol/well, in eight experiments), and, to a much lesser extent, to cardiolipin-coated wells (27 and 44 fmol/well, in two experiments). In contrast, prothrombin binding to phosphatidylcholine-coated wells was only 6.2 and 7.0 fmol/well in two experiments, a figure similar to the value obtained for nonspecific binding to phosphatidylserine-coated wells in the presence of EDTA (6 to 9 fmol/well). As shown in Fig 6A, immunoaffinity-purified patient IgG inhibited the binding of prothrombin to phosphatidylserine-coated wells by 73% and 66%, respec-

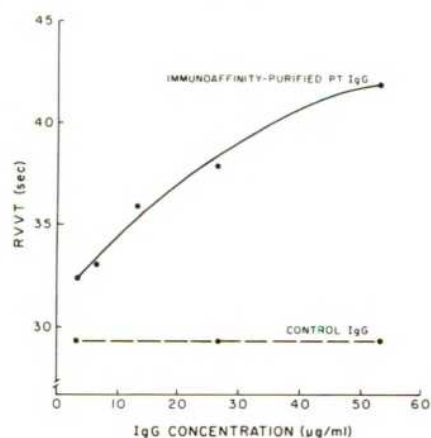


Fig 3. Effect of affinity-purified patient IgG and control IgG on the dilute Russell viper venom time (RVVT) of normal plasma.

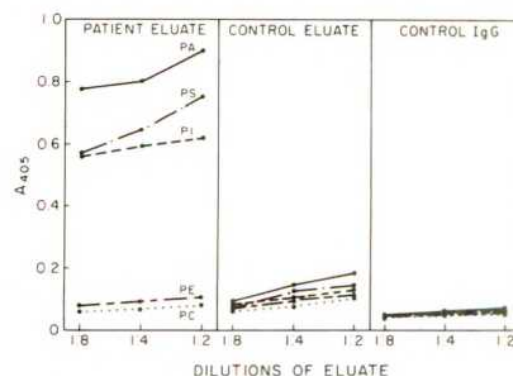


Fig 4. Antiphospholipid activity (ELISA) of cardiolipin liposome eluates. Because control eluates contained essentially no IgG, reactivity of purified normal IgG is also shown.

tively. Purified IgG from patient no. 1 (not shown in Fig 6), tested only at a much lower protein concentration (23 µg/mL), inhibited prothrombin binding by 25.5%. Binding of factor X was also inhibited by patient IgG, although to a slightly lesser extent than prothrombin (Fig 6B). Three control IgG preparations showed no inhibition of prothrombin or factor X binding. Binding to cardiolipin-coated wells was also inhibited by patient eluates.

DISCUSSION

Although earlier observations implied that lupus anticoagulants have as their locus of action the negatively charged phospholipids,^{1,3,5,6,34} this assumption has been directly demonstrated in only one patient.¹² On the basis of this case, the correlation that has been observed between the presence of anticardiolipin activity and lupus anticoagulant activity,⁷⁻¹¹ as well as some more recently described absorption studies,³⁵ all lupus anticoagulants have been widely assumed to operate by the same mechanism. We tested the generality of this hypothesis directly in five patients with lupuslike syndromes and/or thrombosis, whose lupus anticoagulants were largely IgG.

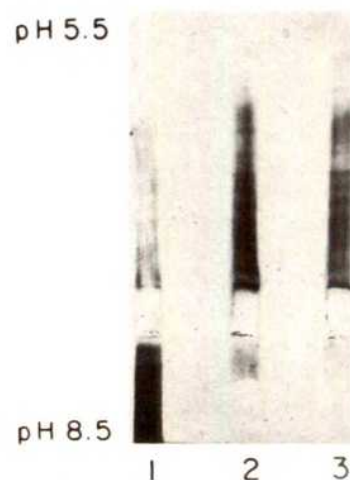


Fig 5. Analytical isoelectric focusing of normal IgG (lane 1) and immunoaffinity-purified IgG from patients no. 2 and 5 (lanes 2 and 3).

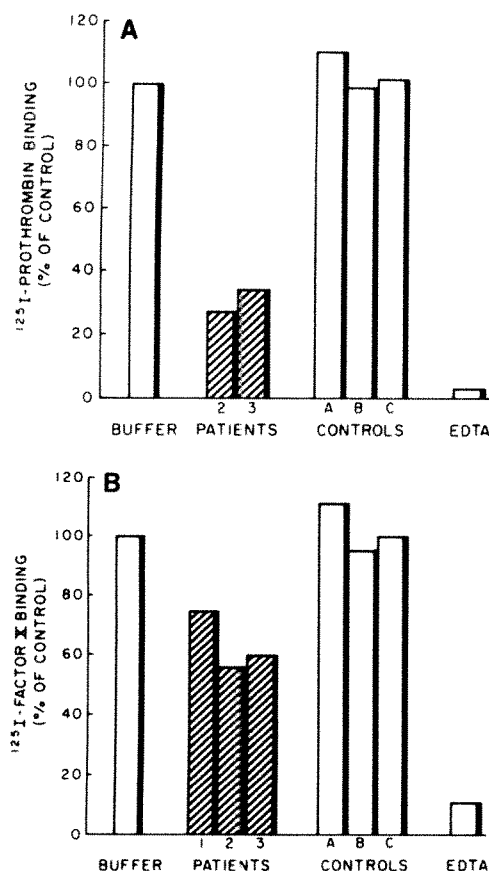


Fig 6. Effect of affinity-purified antibodies on binding of ^{125}I -prothrombin (A) and ^{125}I -factor X (B) to phosphatidylserine-coated wells. Patients are identified by number. Controls are purified normal IgG at the same concentration as affinity-purified patient IgG.

We demonstrated that both lupus anticoagulant and anticardiolipin activities are mediated by the F(ab')_2 portion of IgG and therefore are immunologic in nature. We demonstrated that all, or nearly all, lupus anticoagulant activity can be adsorbed on cardiolipin-containing liposomes, but not on phosphatidylcholine-containing liposomes. We further demonstrated that with a combination of cardiolipin-liposome adsorption, high molarity NaCl and ether elution, and protein A-Sepharose chromatography, it is possible to obtain pure, phospholipid-free, IgG fractions from patient plasma that interact with anionic but not neutral phospholipids and also act as lupus anticoagulants. Recently, Harris and associates¹¹ reported a cardiolipin liposome purification procedure using 1 mol/L of sodium iodide as the elutant. We previously used sodium iodide as an elutant from phosphatidic acid³⁶ and phosphatidylserine³⁷ liposomes, but found that these preparations were heavily contaminated with phospholipid, making some subsequent testing procedures difficult to evaluate. For example, the presence of free and IgG-bound phospholipid in the liposome eluates interfered with coagulation tests and was capable of producing spurious results on isoelectric focusing. For this reason, we developed the present liposome elution procedure. In addition, since

cardiolipin liposome eluates invariably contain significant amounts of albumin, a further purification step by protein A-Sepharose chromatography was added.

Using these preparations, we were able to obtain a good dose-response relationship between lupus anticoagulant activity and IgG concentration and to detect lupus anticoagulant activity at IgG concentrations as low as 3 $\mu\text{g/mL}$. Thus, the dilute RVVT, as normally performed on plasma, can detect lupus anticoagulants at plasma concentrations of $\geq 10 \mu\text{g/mL}$.

The mechanism of prolongation of phospholipid-dependent coagulation tests in the patients we studied is similar to that of the patient previously reported.¹² Our data clearly show inhibition by patient affinity-purified IgG of prothrombin binding to phosphatidylserine surfaces and, to a lesser extent, a similar inhibition of factor X binding. The reason for this difference in inhibition is not presently known. Our data further indicate that prothrombin can bind to cardiolipin surfaces, although to a much lesser extent than to phosphatidylserine surfaces, and that this binding is also inhibited by the affinity-purified IgG.

Although all six lupus anticoagulants we studied in this and the previous¹² report are clearly antibodies with anionic phospholipid specificity, these anticoagulants probably represent only a subpopulation of antibodies to anionic phospholipids. Several studies suggest that there may be more than one type of anticardiolipin antibody,^{10,38-40} and we have studied patients with true-positive and false-positive serologies whose anticardiolipin antibodies did not possess lupus anticoagulant activity. Furthermore, the presence of several distinct bands on isoelectric focusing of the affinity-purified IgG preparations from our patients raises the possibility that not all affinity-purified IgG anticardiolipin antibodies possess lupus anticoagulant activity.

In addition to anticardiolipin antibodies, other phenomena have been correlated with the presence of a lupus anticoagulant, including biologic false-positive serology,^{1,3,40,41} thrombocytopenia,^{7,20} a history of recurrent abortions,⁴²⁻⁵³ and a high incidence of thromboembolic phenomena.^{1-7,42,43} Three of our patients had false-positive serologies, and only 1 may have had thrombocytopenia, whereas 4 of the 5 had either a history of thrombosis or of recurrent abortion. Because this is a highly selected group, no conclusions can be drawn concerning incidence of these clinical findings. Because all patients had anticardiolipin antibodies, it is surprising that two had negative serologies. Even a high concentration (56 $\mu\text{g/mL}$) of affinity-purified IgG from one patient did not produce a positive RPR serology, suggesting that antibody concentration alone may not explain this observation. In this connection, it is of interest that the two patients with positive serologies at the time of our study had some IgM lupus anticoagulant and IgM anticardiolipin activity. Because many flocculation tests are more sensitive to IgM than to IgG antibodies,⁵⁴ a biologic false-positive serology may correlate better with levels of IgM than of IgG anticardiolipin antibodies.

The high correlation between lupus anticoagulants and thrombocytopenia raises the question of the interaction of lupus anticoagulants with platelets. The strong correlation

with thromboembolic phenomena and the possible effect of lupus anticoagulants on endothelial cell prostacyclin production⁴⁷ raise similar questions concerning the interaction of these antibodies with endothelial cells. It will be of interest to determine whether purified lupus anticoagulants interact with platelets or endothelial cells, or whether the clinical phenomena observed depend on other factors.

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Cytoplasmic Factor Required for Entry of Malaria Parasites Into RBCs

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Resealed ghosts of human RBCs, containing diluted cytosol, are susceptible to invasion by *Plasmodium falciparum*. If ATP is present, a dilution of up to about 30-fold, corresponding to an intracellular hemoglobin concentration of ~10 mg/mL, can be tolerated without total loss of susceptibility to invasion. Up to a dilution of about one-half this, the parasites also develop normally. When the cytosol is diluted by more than the critical amount, invasion of the resulting resealed ghosts falls off abruptly. If the diluent buffer is replaced by extraneous concentrated hemolysate, an indefinite dilution is possible without loss of invasion. There is thus an intracellular constituent, which must be present at a concentration above some critical level if the parasite is to enter the cell. The factor in question is not dialyzable. It is largely inactivated when the hemolysate is kept for ~1 day in the cold or for ~20 minutes at 45°C. The inability of a heat-treated hemolysate to support invasion is not due to the generation of inhibitory products, because such a solution can be used as a diluent of a fresh

hemolysate without inhibition of invasion. When the inactivated hemolysate is present as a major component, however, the parasites fail to develop to the trophozoite stage. The invasion-linked factor remains in the strongly adsorbed nonheme fraction when a batchwise separation from hemoglobin on an anion exchanger is made and is thus probably acidic in character; the adsorbed fraction, recovered from the ion-exchanger, substantially restores capacity for invasion when sealed into ghosts. Its activity is destroyed by treatment with trypsin. The adsorbed fraction contains many proteins. When fractionated on a gel filtration column by fast liquid chromatography, active material eluted at a volume corresponding to a mol wt for a globular protein in the region of 10,000. A component of apparent subunit mol wt of 13,000 was observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of this eluate fraction.

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WHITE GHOSTS prepared from simian RBCs do not support invasion by merozoites of *Plasmodium knowlesi*,^{1,2} even though the initial recognition process still appears to operate.¹ It has, however, proved possible to prepare resealed ghosts that are susceptible to invasion by ensuring a sufficient ATP concentration in the internal solution.^{3,4} There are indications that the ATP is required for phosphorylation of membrane-associated proteins, possibly spectrin.⁴ To prepare resealed ghosts, we dialyzed RBCs against a hypotonic medium, including in the solution any solutes to be introduced into the cells; the dialysate is then restored to isotonicity and dialysis is continued at 37° to bring about resealing.² When ATP is present, the hematocrit of the original cell suspension can be reduced to 5% to 10% without eliminating the capacity for invasion of the resulting resealed ghosts; that is, a hemoglobin concentration of <30 mg/mL inside the cell is sufficient to allow invasion and indeed development of the parasites.⁴ However, a dilution is eventually reached at which no invasion will occur. Thus a component in the cytoplasm, other than ATP, is evidently required, the concentration of which must not fall below some critical level, if the membrane is to remain capable of penetration by the merozoite. We report here the results of our attempts to establish the nature of this component.

MATERIALS AND METHODS

P. falciparum was maintained in continuous culture⁵ in adult human RBCs. Schizonts were isolated by a procedure based on that of Saul and colleagues,⁶ involving centrifugation on a cushion of Percoll.⁷

Resealed ghosts, containing 1 mmol/L of magnesium ATP, were prepared as described previously.^{2,4} In brief, washed intact cells were suspended at the required hematocrit in a solution of the required composition and dialysed at 4°C for 2 hours against 100 vol of 5 mmol/L of phosphate buffer, pH 7.4, containing in addition any diffusible solutes present in the suspending solution inside the dialysis bag. The dialysate was then restored to isotonicity (0.15 mol/L of sodium chloride, 5 mmol/L of potassium phosphate), and dialysis was resumed for 45 minutes at 37°C. The resulting resealed ghosts were washed three times in culture medium (RPMI 1640,

containing 10% vol/vol human serum), and were finally suspended at 50% hematocrit in the same solution. The hemoglobin content of the cells was determined by lysing an aliquot after the buffer wash with an equal volume of the 5 mmol/L of phosphate buffer and determining the absorbance at 577 nm, the millimolar absorptivity per heme being taken as 15.2. The Visking dialysis tubing was prepared by boiling in three changes of a solution of 0.1 g/L of sodium bicarbonate and 0.1 mmol/L of EDTA.

Undiluted fresh hemolysate was prepared by freezing and thawing packed washed cells three times and removing stromal material by centrifugation at 42,000 g for 20 minutes. Heat denaturation experiments were carried out by warming the hemolysate in a water bath at the desired temperature for 15 or 20 minutes and clarifying the solution by centrifugation as before. Small volumes were rapidly stripped of dialyzable components on centrifugal columns, packed with Sephadex G-25 in isotonic buffer. The columns (supplied by Pharmacia Fine Chemicals, Uppsala, Sweden) were 10 cm long, and contained ~1.8 mL of Sephadex. After centrifugation of the columns packed with Sephadex at 200 g in a bench-top centrifuge for 2 minutes, 0.1 or 0.2 mL of the hemolysate was applied to each column and brought through by a further centrifugation. Hemoglobin concentrations were determined spectrophotometrically, and ATP was determined by luciferin-luciferase assay,⁸ using an LKB Lumimeter 1250 (LKB Instruments, Bromma, Sweden).

Batch-fractionation of the hemolysate was accomplished on diethylaminoethanol (DE) 52 cellulose (Whatman, Maidstone, UK) following the general procedure of Cheung and colleagues.⁹ The ion-exchanger, equilibrated with 10 mmol/L of sodium phosphate, pH 7.5, was added to a hemolysate (1 g to 5 mL), prepared by lysis of

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packed, washed cells with 2 vol ice-cold water, followed by centrifugation at 42,000 g for 20 minutes to remove stroma. The mixture was gently stirred for 30 minutes at 4°C and centrifuged at low speed. The supernatant, containing the hemoglobin, was removed, and the cellulose was washed with 5 mmol/L of phosphate buffer, pH 7.4. The adsorbed protein was liberated from the cellulose by addition of potassium chloride 1 mL of 0.5 mol/L/g of cellulose, followed by stirring for 1 hour on ice. The supernatant was collected after brief centrifugation and concentrated by passage through a centrifuge tube packed with dry Sephadex G-25.¹⁰ It was finally reequilibrated with isotonic phosphate-buffered saline (PBS), using the centrifugal Sephadex column, as above. A nominal protein concentration was obtained by the method of Bradford,¹¹ and was generally ~1 mg/mL. This preparation could be stored frozen for some days without loss of activity.

A hemoglobin preparation, free of most minor cytoplasmic proteins, was also prepared by chromatography of centrifuged hemolysate on a column (2.5 × 90 cm) of Sephadex G-75.

To examine the effect of proteolysis, trypsin (10 µg/mL final concentration) was added to the solution of nonheme protein, prepared as above. After 5 minutes at 37°, soyabean trypsin inhibitor was added from a stock solution to give a final concentration of 100 µg/mL. As a control, samples were treated with a mixture of trypsin and trypsin inhibitor in the same final proportions.

The first attempt at further fractionation of the active nonheme hemolysate fraction made use of a triacetate ultrafiltration membrane (Sartorius Centrisart; Sartorius, Göttingen, FRG) with a nominal mol-wt cut-off of 20,000. To characterize and isolate the active material, fast liquid chromatography (Pharmacia FPLC) was used, with two Superose 12 columns (1 × 50 cm) in series. Samples of 0.5 mL were applied, and the columns were eluted with PBS, pH 7.4, at a flow rate of 0.7 mL/min. The column was calibrated with the following proteins as mol-wt markers: β -galactosidase, human immunoglobulin G, transferrin, bovine serum albumin (BSA), ovalbumin, trypsinogen, bovine β -lactoglobulin, soy bean trypsin inhibitor, bovine pancreatic ribonuclease, horse heart cytochrome c, and bovine pancreatic trypsin inhibitor. Potassium nitrate was used as a marker for the included volume, and blue dextran (Pharmacia) was used for the void volume. Fractions of 1.5 mL were collected and stored frozen. The fractions were concentrated to 0.3 to 0.4 mL with dry Sephadex G-25, as described. For invasion assays, the material was incorporated into resealed ghosts: the hematocrit was chosen to give a low basal invasion level (Fig 1). Thus, lysis and resealing was done in a mixture containing 12 µL of packed cells (sampled with a positive-displacement pipette), 10 µL of 20 mmol/L of magnesium ATP, 100 µL of the concentrated fraction, made up to 200 µL with buffer; three or four replicate assays were performed on each fraction.

Gel electrophoresis with sodium dodecyl sulfate (SDS) was performed in vertical polyacrylamide slab gels in the system of Swank and Munkres,¹² which gives good resolution in the low-mol-wt range. The gels were stained with Coomassie brilliant blue or silver.¹³ Experiments were also performed with resealed ghosts containing added enzymes, ie, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, phosphofructokinase, catalase, carbonic anhydrase, and superoxide dismutase (Sigma Chemicals, St Louis). These were added to the suspending solution before reversible lysis at the concentration corresponding to that present in the normal RBC.¹⁴

For invasion assays, purified schizonts (80% to 90% purity) were added to the RBC or ghost suspension; after incubation, parasitemia levels were determined by counting 10³ cells on thin smears, stained with Giemsa's solution. Invasion efficiency is expressed in terms of the invasion ratio,⁴ which is defined as the ratio of the proportion of

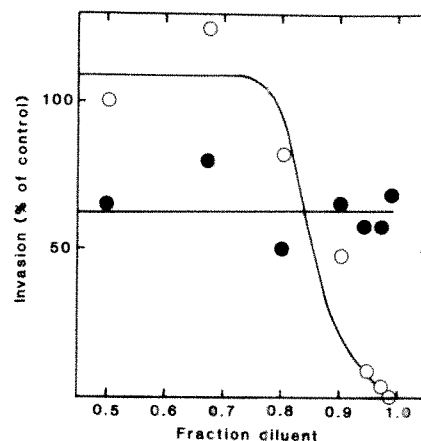


Fig 1. Invasion of resealed ghosts (average of seven experiments), containing the original cytosol, diluted as indicated with isotonic saline buffer (○) and with a fresh hemolysate (●); 1 mmol/L of ATP was present throughout. The controls (100%) were resealed ghosts containing the fresh hemolysate, diluted with an equal volume of buffer, giving an average invasion ratio of 2.2. The point at highest dilution corresponds to a 64-fold dilution (volume fraction of diluent 0.984).

parasitized cells at a given time to that at the time of inoculation (0 hours).

RESULTS

Figure 1 shows the dependence of invasion by merozoites on the dilution of the cell contents. ATP at 1 mmol/L, added as the magnesium salt, was present throughout. There was no major or consistent effect of 2,3-diphosphoglycerate or glutathione (GSH) on the invasion levels, although occasionally (presumably when some oxidation of membrane protein thiol groups had occurred) glutathione increased invasion perceptibly (data not shown). The profile of Fig 1 shows a rather abrupt decline of invasion at a critical dilution of the hemolysate, which varied somewhat from preparation to preparation, but always fell to zero at 20- to 50-fold. In the experiment shown (Figs 1 and 2), there was still detectable invasion at 30-fold dilution (97% diluent buffer in Fig 1), corresponding to an intracellular hemoglobin concentration of ~10 mg/mL. Under these conditions, the staining of the cytosol is so weak that phase contrast is required to see the cells clearly. At a 16-fold dilution (hemoglobin concentration ~20 mg/mL), the parasites continued to develop, and ring forms could be observed, whereas at higher dilution there was little indication of maturation (Fig 2).

The implication of the profile of invasion against dilution, seen in Fig 1, is that a cytosolic component other than ATP, diphosphoglycerate, or glutathione is required for invasion and that its concentration must exceed ~1/30 of its normal concentration in the circulating RBC. This factor may bind to the membrane and be progressively dissociated on dilution. To confirm that an intracellular factor exists, and that the decline of the capacity for invasion is not due to an irreversible change in the membrane, resulting from dilution, the same experiment was made with concentrated freshly prepared hemolysate in place of isotonic buffer. A dilution of

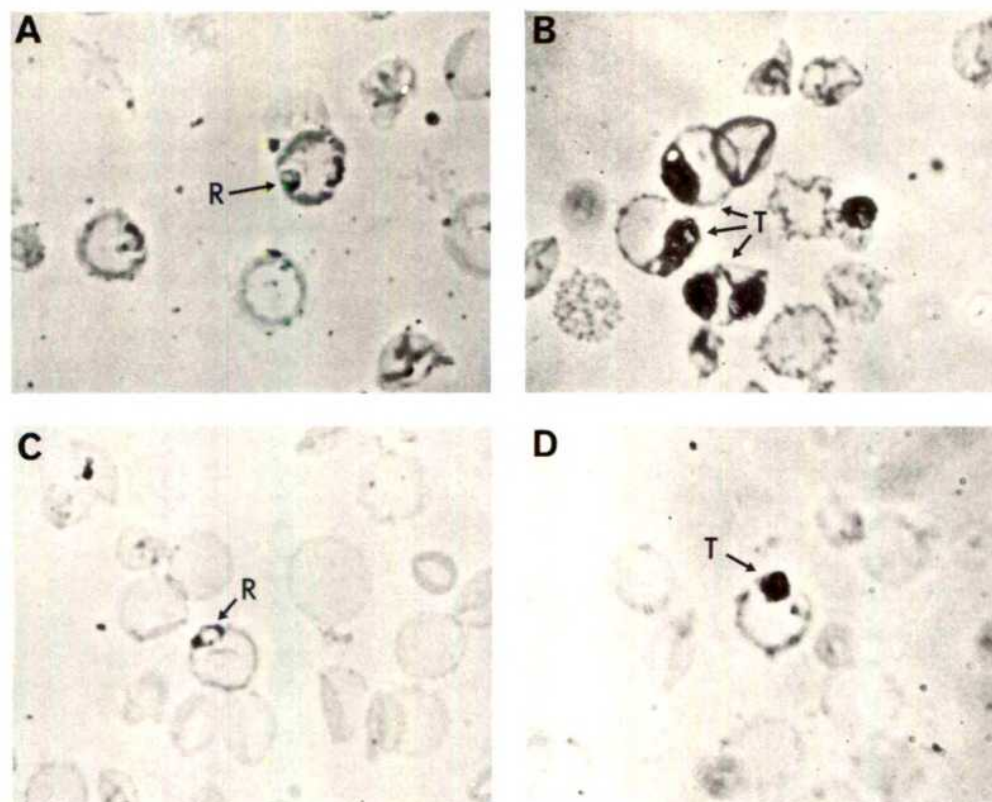


Fig 2. Infected resealed ghosts, viewed in phase contrast (original magnification $\times 1,500$; current magnification, $\times 1,000$). R, rings; T, trophozoites. (A) Ghost contents are cytosol diluted 16-fold with isotonic buffer (hemoglobin concentration ~ 20 mg/mL), 21 hours after inoculation; (B) the same 45 hours after inoculation; (C) ghost contents are cytosol diluted by a factor of 32 (hemoglobin concentration ~ 10 mg/mL) 21 hours after inoculation; (D) the same 45 hours after inoculation. Trophozoites are poorly developed in (D) as compared with (B).

1:1 of the fresh hemolysate (prepared by freezing and thawing of packed cells, followed by centrifugal removal of stromal material) was selected, being more effective in promoting invasion than the undiluted hemolysate. The results of such an experiment are shown in Fig 1, from which it is clear that there is no longer any decline of invasion at some critical dilution of the membranes during lysis and resealing. Thus, an essential constituent for invasion is indeed present in the cytosol. The slightly lower basal level of invasion when undiluted hemolysate was used was always observed and remains unexplained; we conjecture that it may be related to the effect of internal viscosity on the membrane properties.¹⁵

To establish whether the unknown component is a dialyzable factor or a macromolecule, the hemolysate was dialyzed for 24 hours against isotonic buffer containing 1 mmol/L of ATP. When this was used in a lysis and resealing experiment, the resulting cells were poorly invaded. However, a comparable loss of invasion capacity occurred when the hemolysate was simply allowed to stand at 4°C for a similar period. Thus, the factor in vitro is substantially inactivated within ~ 1 day in the cold. The activity could not be restored with 1 mmol/L of glutathione. A rapid method for removal of dialyzable solutes was therefore used. Small centrifugal columns packed with Sephadex G-25 proved satisfactory. About 100

μL of fresh concentrated hemolysate could be applied to each, and the hemoglobin was recovered, diluted by not more than 25%. As judged by analysis of the ATP content of the fresh hemolysate and the centrifugal eluant, removal of ATP and, by implication, of other dialyzable solutes, was complete. This procedure required only a few minutes. The resulting stripped hemolysate retained most of its capacity to restore the invasion of resealed ghosts (Table 1). We infer that the factor is macromolecular.

If the molecule in question is a protein, and is inactivated on standing in the cold, one might expect that it would be sensitive to denaturation generally. The hemolysate was therefore subjected to heat treatment. With increasing temperature, appreciable turbidity was apparent in the solution after dilution for spectrophotometry. Moreover, ghosts resealed with hemolysate, which had been treated at $\geq 50^{\circ}$ for 15 minutes and centrifuged, separated into two layers after sealing, the lower one darker than the upper. Thus, at high temperature, complicating factors arise. However, inactivation of the hemolysate, with respect to its capacity to restore susceptibility to invasion of sealed ghosts, was largely complete after heating at 45° for 15 minutes (Fig 3, inset). Under these circumstances the hemoglobin was undamaged, as judged by its absorption spectrum, which was that of oxyhemoglobin, both in the visible and Soret regions, with no

Table 1. Invasion of Resealed Ghosts Containing Different Hemolysate Fractions

Contents of Ghosts*	Invasion Ratio†	No. of Experiments	Key in Fig 4‡
Dilute Cytosol (16-fold dilution with buffer§)	0.29 ± 0.10 (SD)	16	A
Concentrated cytosol (extraneous hemolysate, diluted with equal volume buffer)	2.10 ± 0.93	13	B
Stripped hemolysate (as previous, but after removal of dialyzable solutes)	1.40 ± 0.16	3	
Heated hemolysate (fresh hemolysate, heated at 45°C and diluted with equal volume buffer)	0.73 ± 0.35	7	C
Unadsorbed fraction from DE 52 (diluted with equal volume buffer)	0.47 ± 0.08	6	D
Adsorbed fraction from DE 52 diluted threefold with heat-inactivated hemolysate	2.00 ± 1.1	4	E
Adsorbed fraction from DE 52 diluted threefold with buffer	1.05 ± 0.24	6	F

*RBCs were lysed and resealed at 6% hematocrit in the specified solutions

†Invasion ratio is parasitemia after one invasion cycle (~24 hours) to that at inoculation (starting parasitemias in range 1% to 5%).

‡Data represented as points in Fig 4.

§Phosphate-buffered saline, containing 1 mmol/L of magnesium ATP.

indication of the formation of a hemichrome or of methemoglobin (which would have revealed itself in a reduction in the ratio of intensities of the visible bands at 577 and 542 nm, and in the appearance of a band at longer wavelengths).¹⁶

The fall in invasion could be interpreted in terms of heat-inactivation of the hemolysate or of the formation of a product of heating (possibly aggregated protein)¹⁷ that could damage the membrane. To test this, mixtures of fresh and heated hemolysates, matched for hemoglobin concentration, were made up and assayed for their activity in restoring invasion. Within the limits of experimental error, dilution of fresh with heated hemolysate caused no greater reduction of

invasion than did dilution with isotonic buffer (Fig 3). We thus conclude that the active factor is destroyed by relatively mild heat treatment. A striking feature of these results, however, was that when the proportion of heated hemolysate was substantial, the parasites, though present in the cells in undiminished numbers, failed to develop to the trophozoite stage. Thus, heat treatment either reduced the concentration of a constituent required for parasite maturation to below some critical level or generated a species toxic to the intracellular parasite. Hemoglobin, freed of most cytoplasmic proteins, introduced into ghosts, together with magnesium-ATP, gave almost no invasion (Table 1 and Fig 4D).

We attempted to obtain an active fraction from concentrated fresh hemolysate by a batch separation on the anion exchanger, DE52 cellulose.⁹ The supernatant from this procedure had low activity with respect to restoration of invasion of resealed ghosts, whereas the adsorbed nonheme fraction, recovered from the ion-exchanger, showed significant activity, both when mixed with an inactivated hemolysate or with diluent buffer and then introduced into ghosts (Table 1 and Fig 4E and F).

As a further indication that the active constituent is a protein, samples were exposed to trypsin (10 µg/mL for 5 to 10 minutes); the reaction was terminated by addition of an excess of soy bean trypsin inhibitor. Such treatment reduced the invasion of cells containing the preparation (with or without additional DE52-stripped hemolysate) by 50% to 60%. Controls, treated with a trypsin-trypsin inhibitor mixture, showed no such reduction.

To examine whether any of the common enzymes of the RBC cytosol might be implicated, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, aldolase, phosphofructokinase, catalase, carbonic anhydrase, and superoxidase dismutase were incorporated in the diluent buffer used to prepare resealed ghosts, at concentrations corresponding to those in the native cell.¹⁴ In no case was the efficiency of invasion affected (data not shown).

As a first step toward a characterization of the active factor, the active fraction from the anion exchanger was passed through a centrifugal cut-off filter, with a nominal mol-wt exclusion of 20,000 for globular proteins. The activity was recovered in the filtrate. A protein of low mol wt was thus indicated.

The active hemolysate fraction was applied to a Superose

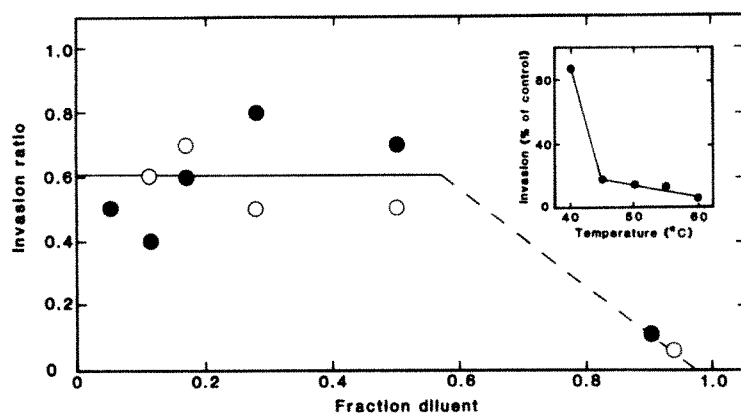


Fig 3. Invasion ratio (ie, parasitaemia ~24 hours after inoculation, relative to that at time of inoculation) in resealed ghosts, containing fresh hemolysate, diluted with varying proportions of isotonic buffer (○) and with heat-inactivated hemolysate (●) (1 mmol/L of ATP present throughout). Inset: Effect of temperature of heating on activity of a fresh hemolysate with respect to its ability to restore susceptibility of resealed ghosts to invasion. The controls were resealed ghosts, containing a 16-fold dilution of hemolysate that had been incubated at 37°C for 15 minutes.

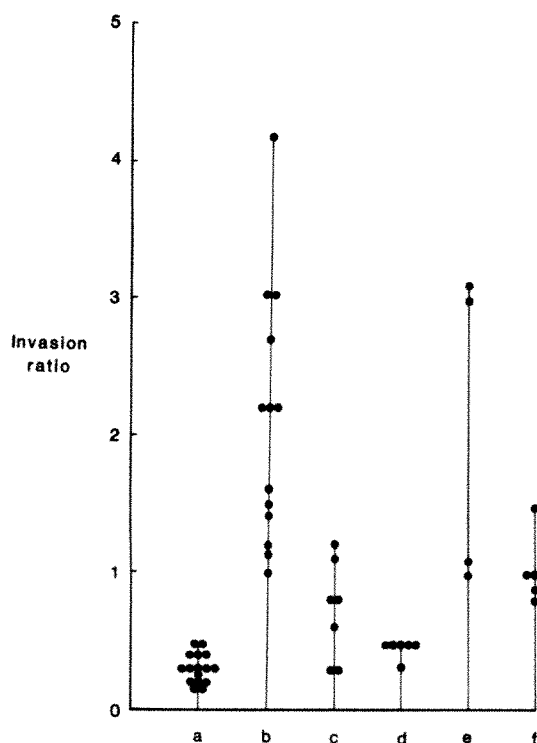


Fig 4. Summary of data for invasion of resealed ghosts, containing various hemolysate-derived preparations: (A) Sixteen-fold dilution of normal contents; (B) dilution 1:1 of freshly prepared hemolysate; (C) dilution 1:1 of heat-treated hemolysate; (D) inactive fraction of hemolysate, recovered after ion-exchange treatment, and containing the hemoglobin; (E) heat-treated hemolysate with added active fraction, recovered from ion-exchanger; (F) 16-fold dilution of normal contents with added active fraction.

12 gel filtration column in an FPLC apparatus. The column was calibrated with a series of globular proteins (Fig 5, inset). Fractions were assayed for activity in promoting malarial invasion. They were first concentrated about four-fold and added to the buffer in which the resealed ghosts were prepared. A hematocrit of 6% (16-fold dilution of cell contents after lysis and resealing) was selected to give a low basal parasitemia (Fig 1). Not all the elution profile could be assayed, because in each of three experiments several fractions caused the resealed ghosts to become misshapen and vesiculate. The reason for this was not discovered; the effect could not be reversed by the addition of 10 mg/mL of fatty acid-free BSA to adsorb lysolipids, or by inclusion of 0.2 mmol/L of EGTA in the buffers used in the course of the preparation up to the column stage, to exclude calcium ions. Three separate elutions allowed assay of most of the profile: only seven fractions (including the void volume) caused vesiculation in all experiments. An elution curve, with activity assays, is shown in Fig 5. In all experiments, activity was discovered within the elution volume range of 38 to 42 mL. This corresponds to a mol wt for a globular protein of ~10,000, although the curvature of the calibration curve in this region¹⁸ limits the precision of the estimate. As Fig 5 also shows, the protein concentration, as judged by the absorbance at 280 nm in this region of the elution profile, is low. Gel electrophoresis in the presence of SDS revealed a component

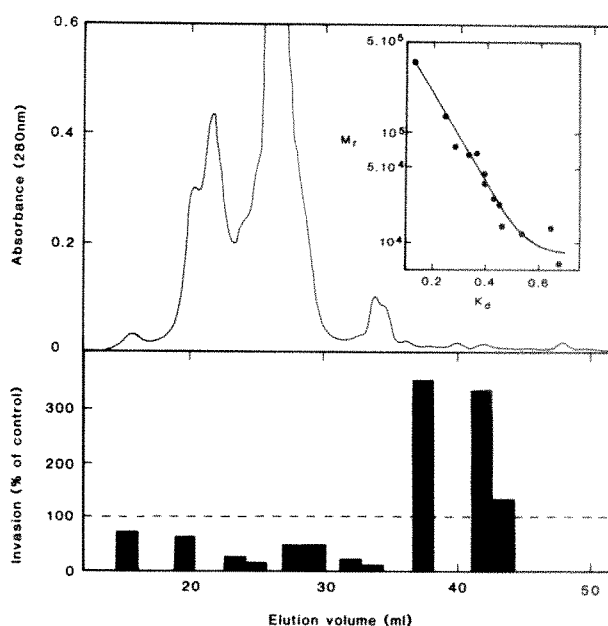


Fig 5. Fast protein liquid chromatography elution profile from Superose 12 column of active preparation, recovered from ion-exchanger. Lower panel shows the activities of the fractions in terms of the invasion of resealed ghosts, into which they had been introduced. Unassayed fractions caused distortion or vesiculation of the ghosts. The controls (100%, corresponding to an invasion ratio of 0.4) were ghosts resealed with a 16-fold dilution of hemolysate with isotonic buffer (containing 1 mmol/L of magnesium ATP), to give a low basal invasion efficiency. Inset: Mol-wt calibration of the column.

with an apparent subunit mol wt of ~13,000 and also various weaker bands.

DISCUSSION

Several unexpected features emerged from these studies. First, resealed ghosts, depleted of the bulk of the hemoglobin and other cytosolic contents, are still susceptible to invasion by *P. falciparum*. Second, only ~6% of the hemoglobin concentration (ie, ~20 mg/mL) is required for the intracellular parasites to develop to the schizont stage. This implies that the amount of hemoglobin needed for the metabolic processes of maturation and egress from the cell is much lower than had been supposed (for review, see ref. 19). After a larger (30-fold) dilution, the capacity of the cytosol to support normal maturation of parasites is altered; dilution of the fresh hemolysate with heat-treated hemolysate has a similar effect. The factor required for maturation could well be a host cell enzyme, such as superoxide dismutase, on which the parasite is thought to be dependent.²⁰

The invasion-related factor must be supposed normally to bind to or to modify the membrane enzymically in such a manner as to affect its structure, as perceived by the parasite at the outer surface. Several RBC proteins bind to the membrane cytoskeleton, including glyceraldehyde 3-phosphate dehydrogenase,²¹ phosphoglycerate kinase,²² phosphofructokinase and aldolase,²³ calmodulin,²⁴ and catalase,²⁵ as well as hemoglobin²⁶; tropomyosin is also associated with the actin protofilaments.²⁷ None of the cytosolic enzymes that we

examined was able to restore the capacity of the diluted cytosol to support invasion, however. In all these cases, dilution, as well as such factors as calcium²⁵ and magnesium²⁶ concentration, may be expected to change grossly the amount of the component bound to the membrane. Whether, and in what manner, different cytoplasmic proteins might affect the state of the membrane is not known at present. The enzymes already tested and hemoglobin can be excluded from consideration. Our results indicate that the factor is a monomeric, anionic protein of low mol wt. Its low stability in vitro may be the result of a tendency to aggregate or adsorb. A very low concentration (<1 µg/mL, as judged by the

staining intensity of the fraction in gel electrophoresis) is required for promotion of invasion. It is thus most likely an enzyme or cofactor. Our earlier evidence^{4,28} suggests that phosphoryl turnover on cytoskeletal proteins, in particular spectrin, may be closely associated with the invasion process, and cytoplasmic as well as membrane-bound kinases or phosphatases are thus likely to be involved.

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Immunophenotyping of Acute Myeloid Leukemia Using Monoclonal Antibodies and the Alkaline Phosphatase–Antialkaline Phosphatase Technique

By Curtis A. Hanson, Kazimiera J. Gajl-Peczalska, Janet L. Parkin, and Richard D. Brunning

The leukemic cells from 41 cases of acute myeloid leukemia (AML) and 17 cases of acute lymphocytic leukemia (ALL) were immunophenotyped by the alkaline phosphatase–antialkaline phosphatase (APAAP) immunocytochemical technique utilizing eight monoclonal antibodies (MoAb) reactive with cells of myeloid origin and seven MoAb reactive with lymphoid antigens. Ninety percent of the cases of AML reacted with one or more of the pan-myeloid MoAb, My7, My9, or 20.3. Reactivity of the myeloid panel of MoAb showed some correlation with the French-American-British (FAB) classification of AML. Five of six cases of acute promyelocytic leukemia (APL) were HLA-DR negative; the one HLA-DR–positive APL had a minor population of HLA-DR–negative promyelocytes. OKM5 and/or My4 reacted with 16 of 16 monocytic leukemias. No specific marker of early erythroid development was identified. AP3, a MoAb reactive with platelet glycoprotein (GPIIIa), was specific for acute megakaryoblastic leukemia. Immunocytochemistry was also helpful in classifying seven cases of

AML with equivocal or negative routine cytochemistry. Two cases of AML had minor populations of blasts detected by the APAAP technique that were immunologically distinct from the major blast population; these minor populations emerged as the predominant cell type at relapse. Two cases of ALL expressed multiple myeloid and lymphoid antigens. Two other cases that morphologically were ALL reacted with only myeloid MoAb; one consisted entirely of immature basophils on ultrastructural examination. Immunophenotyping results using the APAAP technique were comparable with those obtained with flow cytometry. The APAAP technique is a reliable method for immunophenotyping leukemia that complements other methods of immunologic evaluation. The primary advantages of this method include its use with routinely prepared blood and bone marrow smears and the ability to correlate immunocytochemical reactions with morphology.

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THE DIAGNOSIS and classification of acute leukemia is currently based on a combination of morphological,¹ cytochemical,¹ immunologic,² and cytogenetic³ findings. Although the role of immunologic markers is well established in acute lymphocytic leukemia (ALL),⁴ the recent availability of relatively specific myeloid monoclonal antibodies has given impetus to the application of immunophenotyping in the evaluation of acute myeloid leukemia (AML).⁵⁻¹² Diagnostically, this application relates to two primary points: the distinction of AML from ALL, and the relationship of patterns of monoclonal antibody (MoAb) reactivity to the classification of AML.

Immunophenotyping of leukemia and lymphoma cells has been primarily performed on mononuclear cell suspensions by immunofluorescent methods using either flow cytometry or light microscopy.¹³ Recent studies demonstrated the applicability of immunocytochemistry to the immunologic evaluation of hematopoietic malignancies.¹⁴⁻²² Most of these studies have used the immunoperoxidase technique; however, this enzymatic label has limited application for blood and bone marrow specimens because of difficulties in ablating the endogenous peroxidase activity in myeloid cells.²³ The recently developed immunoalkaline phosphatase methodology obviates this difficulty and greatly enhances the use of immunocytochemistry in the immunologic study of both lymphoid and myeloid leukemia.¹⁴⁻¹⁸

The present study describes the results of the application of the alkaline phosphatase–antialkaline phosphatase (APAAP) immunocytochemical technique to the immunophenotyping of 58 cases of acute leukemia, both myeloid and lymphoid, using routinely prepared, air-dried blood and bone marrow smears.

MATERIALS AND METHODS

The case population for this study consisted of 58 patients with acute leukemia diagnosed in the Hematopathology Laboratory at the University of Minnesota Hospital between January 1985 and

March 1986; 41 of the patients were diagnosed as AML, and 17 were diagnosed as ALL. Studies on 32 AML and 12 ALL cases were performed on specimens taken prior to chemotherapy; 9 AML and 5 ALL cases were studied at relapse.

Morphologic classification was based on criteria established by the French-American-British (FAB) Cooperative Study Group.¹ The 41 cases of AML were subclassified as follows: M1, 5 cases; M2, 10 cases; M3, 4 cases; M3 variant, 2 cases; M4, 12 cases; M5a, 1 case; M5b, 3 cases; M6, 3 cases, and M7, 1 case. Bone marrow specimens were prepared according to previously described methods.²⁴ Cytochemical studies included myeloperoxidase (MPO), sudan black B (SBB), and nonspecific esterase (NSE).²⁶⁻²⁸ Terminal deoxynucleotidyl transferase (TdT) determination was performed on all specimens by indirect immunofluorescence.²⁸ Ultrastructural evaluation included routine transmission electron microscopy and myeloperoxidase and platelet peroxidase ultracytochemistry.^{29,30}

Monoclonal antibodies. Sixteen MoAbs that react with cells of myeloid origin were initially evaluated³¹⁻⁴³; the following eight were selected because of their apparent utility in distinguishing AML from ALL and in establishing an immunophenotypic pattern correlating with the FAB classification of AML: HLA-DR, My7, My9, 20.3, My4, OKM5, OKT9, and AP3. Expression of HLA-DR, which is present on multiple cell types including myeloblasts,³¹ is thought to be absent from granulocytes beginning at the promyelocyte stage of differentiation.³² My7,³³ My9³⁴ and 20.3³⁵ are antibodies reactive with most stages of granulocytic and monocytic differentiation; 20.3

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is also expressed on erythroblasts.³⁵ My4³³ and OKM5³⁶ are reactive with cells of monocytic origin; in addition, OKM5 reacts with erythroblasts, platelets, and megakaryocytes.³⁶ OKT9, an antitransferrin receptor antibody,³⁷ reacts with erythroid precursors, thymic lymphocytes, and high mitotic rate tumor proliferations. AP3, a MoAb that recognizes platelet glycoprotein IIIa (GPIIIa),³⁸ can be identified on platelets, megakaryocytes and megakaryoblasts.¹⁸

The other eight myeloid MoAb were not found to add significant differentiation data. Leu M4³⁹ and 20.2³⁵ reacted only with mature granulocytes. Leu M3,³⁹ Leu M1⁴⁰ and OKM1³⁶ identified cells that were morphologically obvious as monocytes, neutrophil myelocytes, or segmented neutrophils. R10,^{41,42} an antiglycophorin A MoAb, was present on late basophilic, polychromatic and orthochromatic erythroblasts but did not react with proerythroblasts and myeloblasts. My10, which is present on hematopoietic stem cells,^{43,44} may be expressed on both lymphoblasts and myeloblasts.

A panel of seven lymphoid monoclonal antibodies, B4,⁴⁵ BA1,⁴⁶ J5,⁴⁷ BA3,⁴⁸ T11,⁴⁹ OKT6⁵⁰ and Leu3,⁵¹ was used to determine B or T cell lineage.

Reagents. Alkaline phosphatase antialkaline phosphatase immunocomplexes were prepared as described by Cordell et al.¹⁴ Calf intestinal alkaline phosphatase (Sigma Chemicals, St Louis) was added at a final concentration of 5 mg/mL to tissue culture supernatant containing mouse monoclonal anti-alkaline phosphatase antibody, which was provided by Dr David Mason, Oxford, England. Unconjugated rabbit antimouse Ig was obtained from Dakopatts (Santa Barbara, CA).

Naphthol-AS-BI, levamisole, fast red TR salt, and trizma base were obtained from Sigma; 0.05 mol/L of Tris-buffered saline (TBS), pH 7.6, was used to dilute all antibodies and the APAAP enzyme complex and was also used as a wash for slides between incubation steps.

Naphthol-AS-BI (2.6 mg) was dissolved in 0.2 mL of dimethyl formamide (Matheson, Coleman, and Bell), followed by the addition of 9.8 mL of 0.1 mol/L of TBS, pH 8.2. Levamisole and fast red TR salt were added at a final concentration of 1 mmol/L and 1 mg/mL, respectively. The substrate solution was then filtered.

Immunocytochemical staining. Immunocytochemical studies were performed on routinely prepared, air-dried blood and bone marrow smears using the APAAP technique after the method of Cordell and colleagues.^{14,18} In brief, slides were fixed in a cold (4°C), 2.5% formalin-acetone mix for 30 to 60 seconds. Primary MoAb was applied for a 30-minute incubation period. Slides were then incubated for 30 minutes with rabbit anti-mouse Ig, the secondary antibody that forms a bridge between the primary MoAb and the APAAP complex, which was then applied for 60 minutes. Following the 60-minute incubation with the APAAP complex, the slides were reincubated with the rabbit anti-mouse antibody for 10 minutes and the APAAP complex for 30 minutes to enhance the intensity of the labeling reaction. This double-bridge procedure for enhancement of the reaction product was described by Vacca.⁵² All incubation procedures were performed in a moist chamber at room temperature and were followed by washing in TBS. The reaction product was developed using the alkaline phosphatase substrate, which was applied for 20 minutes. Slides were then rinsed in tap water, counterstained with Gill's hematoxylin, and coverslipped with glycerin mount. The percentage of positive reacting cells was determined by the assessment of 300 nucleated cells. Positivity with a MoAb was defined as reactivity with >20% of the leukemic cells. There was some variability in the intensity of staining between antibodies and between the different cases. A positive reaction was defined as a cell having an intensity of staining greater than that seen in background residual myeloid cells and in a negative control specimen; equivocal staining was interpreted as a negative reaction. Mouse myeloma ascitic fluid, which contains all immunoglobulin subclasses, was used

as a negative control antibody. The use of fixed cells in the APAAP procedure avoids nonspecific Fc binding problems; incubation of viable cells in solution with antibodies may lead to Fc binding of MoAb in monocytic leukemia with flow cytometric methods.

Thirty-three of the 41 cases of AML and 14 of the 17 cases of ALL were immunophenotyped by flow cytometry using EDTA blood and/or heparinized bone marrow aspirate samples obtained from the same bone marrow procedure as that used for the APAAP study. MoAbs used included HLA-DR and MCS2,⁵³ a myeloid MoAb that recognizes an antigen similar to that identified by My7. The myeloid MoAbs My4, My7, My9, and OKM5 were used in the later stages of the study in 13 cases. Lymphoid MoAbs used in the flow cytometry portion of the study included BA1, BA3, 35.1 (sheep erythrocyte receptor antibody), and OKT6.

RESULTS

Acute myeloid leukemia. Thirty-seven of the 41 cases of AML (90%) reacted with 1 or more of the 3 myelomonocytic antibodies My7, My9, or 20.3 (Table 1); 11 of the cases reacted with all three antibodies. Thirty of the cases (73%) reacted with My7 and 25 (68%) with My9; 88% of the cases reacted with either My7 or My9. Four of the 41 cases did not react with any of these three antibodies; 1 was subclassified as FAB-M1, 2 were classified as M2, and 1 was classified as M7 (megakaryoblastic leukemia).⁵⁴ The case of M7 reacted with AP3, an anti-GPIIIa MoAb. The three cases of non-reacting M1 and M2 were cytochemically positive with MPO and SBB. The one case of M1 had 16% myeloperoxidase-positive blasts; 12% blasts expressed My7, which was lower than our defined threshold of positivity with MoAb. One of the cases of M2 was a therapy-related myeloid leukemia. Myeloid antigen expression was also negative by flow cytometry in the other nonreacting case of M2.

The eight myeloid MoAbs were also evaluated for their ability to discriminate the FAB classification of AML (Table 1). Four immunophenotypic patterns correlated with the FAB subtypes. First, HLA-DR was negative in 5 of 6 cases

Table 1. Cytochemistry and MoAb Reactivity (APAAP) in 41 Cases of Acute Myeloid Leukemia

MoAb	M1 (n = 5)	M2 (n = 10)	M3 (n = 6)	M4 (n = 12)	M5 (n = 4)	M6* (n = 3)	M7 (n = 1)
MPO/							
SBB	3	10	6	9	0	0	0
NSE	0	0	0	10	3	0	0
DR	4	9	1	12	4	2	0
My7	3	6	6	8	4	3	0
My9	2	4(8)†	5(5)	9	3	2	ND
20.3	2	3(8)	3(4)	4(9)	3(3)	3	0
My4	1	4	0	10	4	0	0
OKM5	1	0	0	10	4	0	0
OKT9	2	0(7)	0(3)	0(11)	0	3	0
AP3	0	0	0	0	0	0	1
Leu-3	0(4)	0(8)	0(3)	7	3	0(2)	ND

†Number of cases tested if less than the total number of cases in that classification is shown in parentheses.

Numbers correspond to positive-reacting cases for each FAB type; positivity is defined as MoAb reactivity in >20% of leukemic cells.

Abbreviation: ND, not done.

*Positivity in the erythroleukemias is defined as MoAb reactivity in >20% of the myeloblasts.

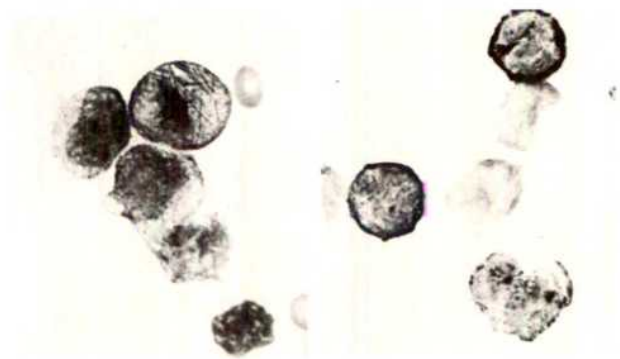


Fig 1. Left: Bone marrow smear from a patient with acute promyelocytic leukemia showing a promyelocyte with multiple Auer rods. Wright's-Giemsa Original magnification $\times 1,000$; current magnification $\times 500$. Right: Three HLA-DR-positive promyelocytes. Alkaline phosphatase-antialkaline phosphatase (APAAP), hematoxylin-counterstain Original magnification $\times 1,000$; current magnification $\times 500$.

of acute promyelocytic leukemia (M3), including 2 cases of the hypogranular variant. The one positive HLA-DR case (Fig 1) consisted of a proliferation of typical-appearing promyelocytes with extensive coarse granulation; cells with multiple Auer rods were identified. Although the majority of promyelocytes in this case were HLA-DR positive, 23% of the promyelocytes were HLA-DR negative. HLA-DR reactivity was present in the majority of cases of AML-M1 and M2 (13 of 15) and in all the monocytic leukemias. Less than 20% of the blasts in two cases of M6 and in the one case of M7 were positive with HLA-DR. Second, all monocytic leukemias reacted with My4 and/or OKM5. In addition, five cases of M1 and M2 showed My4 reactivity on 26% to 52% of the leukemic cells. OKM5 positivity was also detected on erythroblasts and megakaryocytes. Third, OKT9 reacted with erythroblasts, including proerythroblasts, and some myeloblasts in the erythroleukemias; myeloblasts in two cases of M1 also reacted with OKT9. Fourth, AP3 was a specific marker for M7. A positive reaction with AP3 was only observed in the platelets, megakaryocytes, micromegakaryocytes, and megakaryoblasts. Blasts in the other sub-

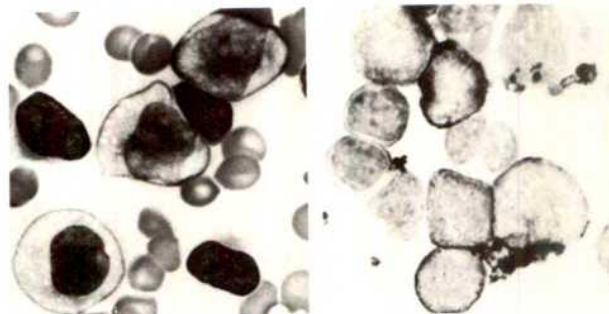


Fig 2. Left: Bone marrow smear from a patient with acute monoblastic leukemia showing several monoblasts. Nonspecific esterase staining was negative. Wright's-Giemsa Original magnification $\times 1,000$; current magnification $\times 500$. Right: Several OKM5-positive monoblasts mixed with negative staining cells. Alkaline phosphatase-antialkaline phosphatase (APAAP), hematoxylin counterstain Original magnification $\times 1,000$; current magnification $\times 500$.

types were uniformly negative with AP3. The one case of M7 was confirmed with platelet peroxidase ultracytochemistry.

Immunophenotyping was also compared with routine cytochemistry. Three cases classified as M1 by morphology were cytochemically equivocal or negative with MPO and SBB and showed positive reactivity with one or more of the myeloid MoAbs My7, My9, or 20.3; one was positive with OKM5 and My4, suggesting a monocytic component. Two cases of M4 and one of M5a (Fig 2) had equivocal or negative staining of the leukemic blasts with NSE. The blasts from all three cases reacted with My4 and OKM5, confirming the monocytic lineage; all cases had monocytic features by electron microscopy.

Two cases of AML-M6 had minor populations of megakaryoblasts and monoblasts, respectively, identified immunocytochemically at diagnosis. In both cases, the minor population emerged as the predominant leukemic cell type at relapse, megakaryoblastic in one (Fig 3) and monoblastic in the other.

None of the 41 cases of AML showed reactivity with the B lineage MoAb. Ten cases (24%) reacted with Leu-3, which is

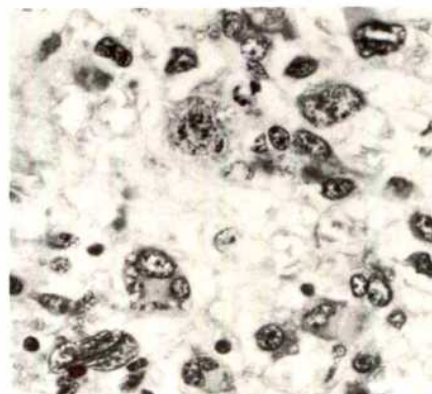
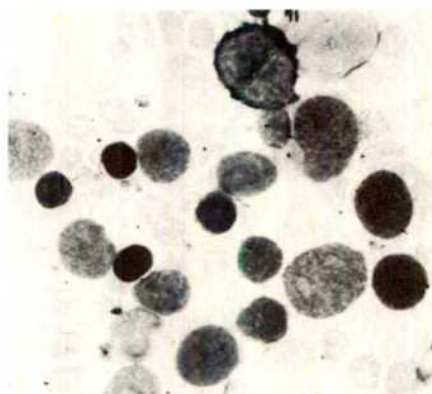


Fig 3. Left: Bone marrow smear from a patient with acute erythroleukemia showing a single blast (arrow) staining with AP3, a monoclonal antibody (MoAb) to platelet glycoprotein IIIa. Alkaline phosphatase-antialkaline phosphatase (APAAP), hematoxylin counterstain Original magnification $\times 1,000$; current magnification $\times 500$. Right: Bone marrow trephine section from the same patient 2 weeks later, postinduction chemotherapy, with numerous atypical megakaryocytes and increased fibrosis. Hematoxylin and eosin Original magnification $\times 400$; current magnification $\times 200$.

thought to recognize a cytoplasmic antigen present in monocytes/macrophages.⁵⁵ The intensity of staining was less than that usually found on Leu-3-positive helper T cells. Ten AML cases reacted with Leu-3; seven were FAB-M4 and three were FAB-M5.

Acute lymphocytic leukemia. Fourteen of the 17 cases of ALL reacted with one or more of the B cell lineage MoAb; one case exhibited a T cell phenotype. Four cases of ALL showed unusual immunophenotyping with the MoAb (Table 2). Virtually all blasts in two cases (nos. 1 and 2) reacted with both lymphoid (B lineage) and myeloid MoAb. A high percentage of the leukemic cells in case 1 had MPO-positive granules by ultracytochemistry; chromosome studies showed a normal 46XY karyotype. Electron microscopic evaluation of case 2 showed undifferentiated blasts with negative ultracytochemistry, compatible with an ALL; cytogenetic studies showed a 47XY,+21 karyotype. The intensity of staining with the lymphoid and myeloid MoAb did not differ in these two cases.

Two cases (nos. 3 and 4) classified as ALL by morphology and TdT positivity were negative with all the lymphoid MoAbs; both reacted with multiple myeloid MoAb (Fig 4). Ultrastructurally, case 3 consisted entirely of immature basophils (Fig 5); basophilic granules were not apparent by light microscopy. Cytogenetic studies showed a 46XY karyotype. Case 4 occurred in an 85-year-old man who had a leukocyte count of $446 \times 10^9/L$ and died within 12 hours of admission. No chromosome analysis was performed.

Correlation of immunocytochemistry with flow cytometry. There was good correlation of the immunophenotype results in the 47 cases evaluated with both the APAAP and flow cytometry techniques; there were no discrepancies in the classification of the leukemias between the two methods. Minor variations in the percentage of reacting cells were sometimes noted and were probably related to the different methods of specimen preparation.

DISCUSSION

The utility of MoAbs in the diagnosis and classification of malignancies of the hematopoietic system is well established.^{2,4-8} The initial studies with these markers primarily used flow cytometry or microscopic immunofluorescent techniques. The introduction of immunocytochemistry has considerably expanded the potential application of immunophenotyping acute leukemia. This increased utility relates primarily to the application of immunocytochemical methodology to routinely prepared blood and bone marrow smears and cytospin preparations.¹⁴⁻²¹ Immunocytochemistry allows

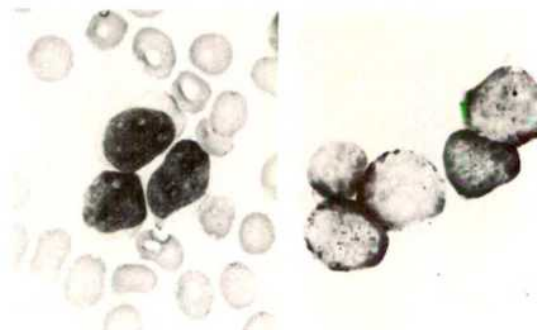


Fig 4. Left: Bone marrow smear from a patient with acute lymphocytic leukemia showing lymphoid-appearing blasts. Right: Blasts from same patient showing My7-positive staining. Alkaline phosphatase-antialkaline phosphatase (APAAP), hematoxylin counterstain. Original magnification $\times 1,000$; current magnification $\times 500$.

direct visualization of the reactivity pattern of individual leukemic cells with the MoAb.

The diagnostic utility of MoAb in the evaluation of cases of acute leukemia, either by flow cytometry or immunocytochemistry, relates to two primary points: the distinction between AML and ALL and the correlation of patterns of antibody reactivity with the classification of AML. In addition,

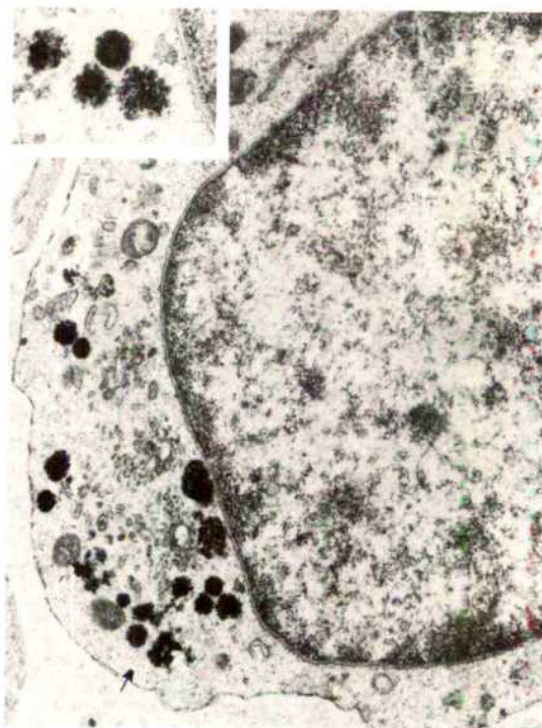


Fig 5. Electron micrograph of a peripheral blood leukemic blast from the same case as shown in Fig 4 illustrates peroxidase-positive granulation (arrows). Peroxidase activity is lacking in the nuclear envelope and rough endoplasmic reticulum. Lead citrate only. Original magnification $\times 19,000$; current magnification $\times 12,350$. Inset: At higher magnification, the peroxidase-containing granules show a speckled pattern of positivity typical for basophil granules. Original magnification $\times 39,000$; current magnification $\times 25,350$.

Table 2. Four Cases of ALL With Unusual Immunophenotype

Case	DR	B4/BA1	CALLA	OKT6/T11	My7	My9	20.3
1	+	+	+	-	+	+	+
2	+	+	+	-	+	ND	+
3	+	-	-	-	+	+	+
4	-	-	-	-	-	+	+

My4, OKM5, OKT9, and AP3 were negative in all cases studied. Abbreviation: ND, not done.

tion, MoAbs make possible more ready recognition of proliferations of more than one cell line in leukemias of mixed lineage.

The use of MoAbs in the evaluation of cases of AML is of more recent origin than in ALL. Because of the several possible differentiation patterns in the myeloid system, the results have not been quite as distinct as in the lymphocytic leukemias.⁵⁻¹¹ A study of 196 AML patients from the Cancer and Leukemia Group B (CALGB) showed My9 reactivity in 70% and My7 expression in 57% of cases.⁷ Neame and colleagues found 87% of 75 cases of AML reacted with My9 and 75% with My7; 96% expressed My9 and/or My7.⁵⁶ My7 and My9 also appear to be highly specific for myeloid leukemias. Griffin and co-workers found reactivity in only 1 of 109 cases of ALL with My7 and 2 of 109 with My9.³⁴

The results from the present study demonstrate that, as in flow cytometry techniques, multiple pan-myeloid MoAb must be used in the evaluation of acute leukemias with immunocytochemical methodologies; 88% of the cases of AML in this series was positive with My7 and/or My9. The MoAb 20.3, which recognizes an antigen present on immature myeloid cells, monocytes, and erythroid cells,³⁵ had a pattern of reactivity similar to that of My7 and My9. The blasts from three cases of AML did not react with any of the myeloid or lymphoid MoAb; MPO and SBB were positive in all three cases. These results underscore the importance of cytochemistry in the classification of AML.

None of the lymphoid MoAbs used in this study except Leu-3 reacted with any of the cases of AML. The lymphoid MoAb identified all but two cases of ALL diagnosed by morphology and TdT positivity; the blasts from both cases reacted with myeloid MoAb. One consisted of immature basophils on electron microscopy. The other case occurred in an elderly male with a marked leukocytosis; no ultrastructural or chromosome analysis was available.

The blasts from two cases of leukemia classified as ALL reacted with both myeloid and lymphoid MoAb. This pattern appears to be an infrequent finding, although some authors have reported 20% to 25% of cases of ALL and AML with mixed immunophenotypes.⁵⁷⁻⁵⁹ The significance of these findings is not clear; it has been reported that cases of ALL that react with myeloid MoAb have a poor response to induction chemotherapy.⁶⁰ Both of the two cases in this study entered remission after the first course of induction chemotherapy.

The reactivity of Leu-3, a marker for helper T cells, with cells of monocytic origin has been previously described.⁵⁵ Wood and associates showed that the normal expression of the Leu-3 antigen on monocytes appears to result from endogenous synthesis and is predominantly cytoplasmic in location.⁵⁵ The APAAP technique, as opposed to routine flow cytometric methods, will detect such cytoplasmic expression as shown in this study.

Studies have shown that myeloid MoAb can be used to subclassify cases of AML.⁵⁻¹² Using a large panel of MoAbs, Neame and colleagues found correlation of the immunophenotype and FAB classification in >80% of 75 cases of AML.⁵⁶ Our results demonstrate that some correlation of immunophenotype with the FAB classification. The lack of

reactivity of HLA-DR in the majority of cases of APL confirms previous observations that the promyelocyte is predominantly HLA-DR negative.^{5-8,32} One case of APL in the current study was HLA-DR positive; however, a small population of HLA-DR-negative promyelocytes was morphologically evident with use of the APAAP technique. This is consistent with the 27% of cases of APL with HLA-DR positivity in the CALGB report.⁷ In contrast to the CALGB finding of HLA-DR expression in two of three hypogranular variants of APL,⁷ the two hypogranular variants in the present study were HLA-DR negative. HLA-DR negativity was not exclusive to APL in the present series since 13% of M1 and M2 cases and 24% of cases of ALL were HLA-DR negative.

The antigens identified by My4 and OKM5 are expressed primarily in M4 and M5 AML; 14 of 16 cases of M4 and M5 were positive with My4 in this study. OKM5 is a MoAb reactive with monocyte determinants and is also expressed on platelets and nucleated RBCs.³⁶ OKM5 was present in 14 of 16 (88%) cases of monocytic leukemia in this study; no other subset showed blast reactivity. The reactivity of OKM5 with platelets, megakaryocytes, and normoblasts did not lead to interpretive difficulties because these cells could be morphologically distinguished with the APAAP technique. OKM5 positivity appeared to begin at the basophilic erythroblast stage; the proerythroblasts and myeloblasts in the erythroleukemias were OKM5 negative. Although some micro-megakaryocytes reacted with OKM5, no megakaryoblasts in the one case of M7 reacted.

No specific and sensitive marker of early erythroid development was identified. R10, an antiglycophorin A antibody,⁴² was found only on cells that were morphologically obvious as erythroblasts beginning at the basophilic stage; no proerythroblast or myeloblast reactivity was noted. This is consistent with other studies showing that this MoAb labels only post-CFU-E stages of development.⁴² As a result, R10 would be of limited value in cases of M6 with markedly immature RBC precursors. OKT9, a MoAb to the transferrin receptor,³⁷ was present on some of the myeloblasts in addition to all the erythroblasts in the erythroleukemias. Blast reactivity with OKT9 was also found on myeloblasts in two cases of M1, however, and may be detected in some cases of ALL.

Previous reports showed that MoAbs against GPIIb/IIIa or IIIa have utility in the diagnosis of M7.^{16,18} The ultrastructural detection of platelet peroxidase is probably the earliest marker of megakaryocytic differentiation and remains the diagnostic standard.^{30,61} Immunologic analysis with MoAb allows more rapid identification, however, at less expense.

Although antibody reactivity with anti-factor VIII antibodies may be observed in some cases of megakaryoblastic leukemia,²² reactivity with MoAb to GPIIb/IIIa is a more sensitive marker of megakaryoblasts than is reactivity with anti-factor VIII.⁶¹

Although immunophenotyping may provide helpful confirmatory data in the classification of AML, the majority of cases in this study were readily classified on the basis of routine morphology and cytochemistry. All seven cases of AML with negative or equivocal routine cytochemistry

reacted with one or more of the myeloid MoAbs, however. These findings suggest that immunocytochemistry may be an important tool for identifying blasts of granulocytic or monocytic lineage when routine cytochemistry is negative.

The APAAP technique also facilitates morphological identification of immunologically heterogeneous cell populations. Two cases of untreated AML had low numbers, <5%, of blasts that were immunocytochemically different from the major population. The minor populations eventually emerged as the predominant cell type at relapse. Detection of such minor populations of leukemic cells may not be possible with flow cytometry because most MoAbs are not leukemia-restricted and react with normal myeloid elements.

This study and another report from this laboratory¹⁸ show that results obtained with APAAP immunocytochemistry are comparable to those obtained with flow cytometry; variations in percentages between methods occur in individual cases. Immunocytochemistry offers advantages over flow cytometry in that routinely prepared blood and marrow smears and cytospin preparations may be used; this is useful when retrospective immunophenotyping may be desirable or when specimens and instrumentation for flow cytometry are not available. Interpretation of results is similar to the

interpretation of myeloperoxidase or nonspecific esterase cytochemistry.

Use of APAAP immunocytochemistry requires strict adherence to high technical standards. The quality of reagents and antibodies must be assured, as in any immunophenotyping procedure; positive and negative controls must be evaluated in all specimen batches. The use of a dilute (2%) formalin-acetone mix for a short time (30 seconds) gives excellent cytomorphology of the cells and maintains surface and cytoplasmic antigenicity. Although antigenicity is usually maintained for several months if slides are stored with dessicant at room temperature (personal observation) or frozen at -20°C ,¹⁴ careful evaluation of controls is necessary when using stored material. Nonspecific reactivity must also be assessed; alkaline phosphatase activity in neutrophils can be effectively inhibited by levamisole.¹⁴ Endogenous phosphatase activity in macrophages can be diminished by maintaining an alkaline reaction (pH 8.2).¹⁴

Immunocytochemistry with the APAAP technique is a relatively new but reliable procedure and is an important addition to currently used methods for evaluating acute leukemia.

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The Gene Located at Chromosome 18 Band q21 Is Rearranged in Uncultured Diffuse Lymphomas as Well as Follicular Lymphomas

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The karyotypic abnormality $t(14;18)(q32;q21)$ is reported to occur in 75% of follicular lymphomas. This translocation results in the rearrangement of a putative oncogene *bcl-2*, which resides at chromosome 18 band q21 (the 18q21 gene). Using two human genomic DNA fragments cloned from the chromosome 18 band q21 as probes, we analyzed 65 uncultured human lymphoma samples by the Southern blot technique. The 18q21 gene was rearranged in 18 of 26 (69%) follicular lymphomas, 3 of 5 (60%) follicular lymphomas transformed to large cell lymphomas, 8 of 20 (40%) diffuse large cell lymphomas (DLCLs), and 2 of 7 (29%) small noncleaved cell lymphomas (SNCs). Our analysis detected rearrangement of the 18q21 gene in 10 of 13 (77%) cases in which the $t(14;18)(q32;q21)$ translocation

was found by cytogenetic techniques. Our analysis also proved helpful in difficult karyotyping situations: (a) identifying the donor chromosome fragment as chromosome 18 band q21 in 4 of 9 (44%) cases that cytogenetically displayed a 14q+ chromosome of unknown origin, and (b) identifying a rearrangement of chromosome 18 band q21 in 12 of 18 (67%) cases that cytogenetically yielded no cells in metaphase. We also demonstrated three cases of submicroscopic rearrangement of the 18q21 gene. In our studies, patients with DLCLs and rearrangement of the 18q21 gene had a significantly higher incidence of extranodal involvement when compared with patients with DLCLs and no 18q21 gene rearrangement ($P = 0.03$).

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RECENT DEVELOPMENTS in cytogenetics and molecular genetics have provided us with a better understanding of the potential genetic mechanisms of tumorigenesis. Several consistent karyotypic abnormalities have been observed in human B cell lymphomas.¹⁻³ Some of these somatic mutations involve reciprocal translocations of a protooncogene and an immunoglobulin gene. About 75% of Burkitt's lymphomas cytogenetically display the $t(8;14)(q24;q32)$ translocation, and the remaining 25% of cases show either the $t(2;8)(p11;q24)$ or the $t(8;22)(q24;q11)$ translocation.⁴ The common feature of these translocations is the involvement of chromosome 8 band q24 where the *c-myc* oncogene resides.⁵ Breakpoints on the counterpart chromosomes occur within the chromosome 14 band q32, chromosome 2 band p11, and chromosome 22 band q11 where the immunoglobulin heavy chain gene, κ light chain gene and λ light chain gene, respectively, reside.^{6,7} The role of the immunoglobulin genes in these instances is still controversial. In vitro studies have shown that the *c-myc* oncogene in these situations is dysregulated and overexpressed.⁸⁻¹⁰ Transgenic mice bearing the *c-myc* oncogene coupled to the immunoglobulin enhancer sequence frequently develop a fatal lymphoma.¹¹

A similar karyotypic abnormality, $t(14;18)(q32;q21)$, has been observed in ~75% of follicular lymphomas, which as a group are one of the most common human B cell malignancies. By analogy, a putative oncogene *bcl-2*, residing at chromosome 18 band q21, has been postulated. Recently, human genomic DNA fragments from this region were cloned from lymphoid cell lines carrying the $t(14;18)(q32;q21)$ translocation by three different groups of investigators.¹²⁻¹⁴ Using these probes, these investigators demonstrated that the gene located at chromosome 18 band q21 (the 18q21 gene) is rearranged in ~60% of follicular lymphomas.¹³⁻¹⁵

This report explores the extent of the rearrangement of this putative oncogene in all forms of human malignant lymphomas simultaneously with cytogenetic analysis, particularly on uncultured cells obtained directly from tumor biopsies or aspirates.

MATERIALS AND METHODS

Procurement of tissues. Fresh uncultured tissues were obtained from diagnostic specimens from 65 patients at The University of Texas MD Anderson Hospital and Tumor Institute at Houston, Houston, Texas (32 lymph node biopsies, 25 lymph node aspirates, 3 bone marrow aspirates, 1 bone biopsy, 1 peripheral blood, 2 pleural fluid aspirates, and 1 ascitic fluid aspirate). All of these samples were reviewed by a member of the hematopathology section and the cytopathology section in the department of pathology and proved to be involved by lymphoma.

Tissue typing. All 65 samples were classified according to the Working Formulation of non-Hodgkin's lymphomas for clinical usage.¹⁶ The determination of follicular or diffuse architecture in the samples of lymph node aspirates was based on the histopathology of the same lymph node whenever possible or on a lymph node biopsy from another site. A subset of patients who displayed follicular large cell lymphoma (FLCL) or diffuse large cell lymphoma (DLCL) histologically, but who initially were diagnosed as having follicular small cleaved cell lymphoma (FSC) or follicular mixed small cleaved and large cell lymphoma (FM), were categorized as transformed follicular lymphoma. The tumor immunophenotype was determined by using one or more markers including IgG, IgM, κ light chain, λ light chain, B1, J5, Leu-1, Leu-2, Leu-3, Leu-4, T11, etc. as described.¹⁷

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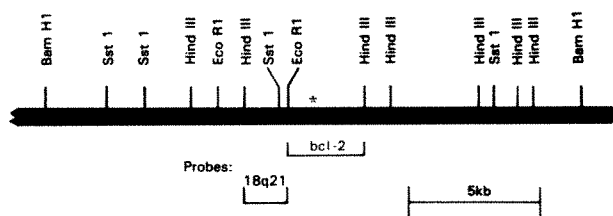


Fig 1. Partial restriction map of chromosome 18 band q21 and probes used. Top bar represents the germline DNA structure of chromosome 18q21. Horizontal lines under chromosome 18 indicate the probes 18q21 and *bcl-2* used; *t(14;18) breakpoint hot spot²⁴⁻²⁶.

Table 1. Configuration of 18q21 Gene in 65 Uncultured Human Lymphomas

Case No.	Karyotype	<i>bcl-2</i> Probe					18q21 Probe				
		<i>Bam</i>	<i>Hind</i>	<i>Rl</i>	<i>Pst</i>	<i>Sst</i>	<i>Bam</i>	<i>Hind</i>	<i>Rl</i>	<i>Pst</i>	<i>Sst</i>
Follicular small cleaved cell											
1	t(14;18)	R	R	—	—	R	—	R	—	—	—
2	t(14;18)	R	G	G	—	R	R	G	G	—	G
3	t(14;18)	G	G	G	—	G	G	G	G	—	G
4	t(14;18)	G	—	G	G	—	G	G	G	G	—
5	14q+	R	R	R	—	R	—	R	G	R	G
6	14q+	G	G	G	—	—	G	G	G	—	—
7	14q+	—	G	—	—	—	—	G	—	—	—
8	NM	—	R	R	—	R	—	R	G	—	—
9	NM	R	R	—	—	G	R	R	—	—	—
10	NM	R	—	—	—	R	—	—	—	R	G
11	NM	R	R	—	—	R	—	—	—	R	G
12	NM	R	—	—	—	R	—	—	—	R	G
13	NM	R	—	—	R	R	—	—	—	—	—
14	NM	—	R	—	—	—	—	G	—	—	—
15	NM	G	G	G	—	—	G	G	G	—	—
16	ND	G	G	G	—	—	G	G	G	—	—
Follicular mixed small cleaved and large cell											
17	t(14;18)	R	R	—	—	G	—	R	—	R	—
18	t(14;18)	R	—	—	—	R	—	R	—	R	G
19	t(14;18)	R	R	—	—	R	—	R	—	R	G
20	t(14;18)	R	—	—	G	R	—	—	—	—	—
21	t(14;18)	—	R	—	—	—	—	G	—	—	—
22	NM	—	—	—	—	—	—	R	—	R	—
23	ND	R	R	—	—	R	—	—	—	R	G
Follicular large cell											
24	t(14;18)	G	—	—	—	G	G	—	—	R	G
25	ND	G	G	G	—	G	G	G	—	G	—
26	ND	G	G	—	—	—	G	G	—	—	—
Transformed follicular lymphoma											
27	NM	R	R	—	—	R	R	R	—	R	G
28	NM	—	G	—	—	—	—	R	—	—	—
29	ND	R	G	R	R	R	—	G	—	R	G
30	ND	G	G	G	G	—	G	G	—	G	—
31	ND	G	G	—	G	G	G	G	—	G	G
Diffuse large cell											
32	t(14;18)	R	R	—	—	—	R	R	—	—	—
33	t(14;18)	R	—	—	—	G	—	R	—	—	R
34	14q+	R	—	—	—	R	—	R	—	R	G
35	14q+	R	R	R	—	—	G	G	—	—	—
36	14q+	R	G	—	—	—	R	G	—	—	—
37	14q+	G	G	G	—	—	G	G	G	—	—
38	14q+	G	—	—	G	G	G	—	—	G	G

Table 1. Configuration of 18q21 Gene in 65 Uncultured Human Lymphomas (Cont'd)

Case No.	Karyotype	<i>bcl-2</i> Probe					18q21 Probe				
		<i>Bam</i>	<i>Hind</i>	<i>Rl</i>	<i>Pst</i>	<i>Sst</i>	<i>Bam</i>	<i>Hind</i>	<i>Rl</i>	<i>Pst</i>	<i>Sst</i>
39	14q+	G	—	—	G	G	G	—	—	G	G
40	—8,—17,etc.	R	—	R	—	—	—	—	G	—	—
41	t(8;14)	—	—	—	—	—	G	G	G	—	—
42	t(11;14)	G	—	—	—	G	G	—	—	—	G
43	t(2;4)	G	G	G	—	—	—	—	—	—	—
44	6q—	G	G	—	G	G	G	G	—	G	—
45	NM	R	R	—	—	—	R	G	—	—	—
46	NM	G	R	R	—	—	G	R	G	—	—
47	NM	G	—	—	—	G	G	—	—	G	G
48	NM	G	G	—	—	—	G	G	—	—	—
49	ND	G	G	—	G	G	G	G	G	—	—
50	ND	—	G	—	—	—	—	G	—	—	—
51	ND	—	G	—	—	—	—	G	—	—	—
Small noncleaved cell											
52	t(14;18)	G	G	—	G	G	G	G	G	G	G
53	t(8;14)	G	G	G	—	—	G	G	G	—	—
54	t(8;14)	G	—	R	—	—	—	—	R	—	—
55	t(8;22)	R	—	R	—	—	—	—	G	—	—
56	t(8;22)	G	G	G	—	—	G	G	G	—	—
57	ND	G	G	G	—	—	G	G	G	—	—
58	ND	—	—	—	—	—	G	G	G	—	—
T cell lymphoma											
59	iso(18q)	G	G	G	G	—	G	G	—	G	G
60	+3	G	—	G	G	G	G	G	—	G	G
61	NM	G	G	G	G	—	G	G	—	G	—
62	NM	—	—	G	G	—	G	G	—	G	—
63	ND	G	G	G	—	—	G	G	G	—	—
Small lymphocytic											
64	46,XY	G	—	—	—	G	G	G	—	G	—
65	ND	G	G	—	G	—	G	G	—	—	—

Abbreviations: NM, no metaphases; ND, not done; *Bam*, *Bam*HI; *Hind*, *Hind*III; *Rl*, *Eco*RI; *Pst*, *Pst*I; *Sst*, *Sst*I; G, germline; R, rearrangement.

Preparation of DNA and Southern blot analysis. High-mol-wt DNAs were prepared by a modification of the method described previously.¹⁸ The DNAs were digested with various restriction endonucleases, size-fractionated in 0.8% agarose gels by electrophoresis, and then transferred onto nitrocellulose filters as described by Southern.¹⁹

Probes were nick-translated with [α -³²P] to a specific activity of 1 to 3 $\times$ 10⁸ cpm/ μ g of DNA. Hybridization was carried out at 42°C overnight in the presence of 10% dextran sulfate. The filters were then extensively washed with 0.1% sodium dodecyl sulfate (SDS)/0.1X sodium chloride and sodium citrate (SSC) at 65°C. Autoradiography was done at -70°C with intensifying screens.

Probes used. Two human genomic DNA fragments located at chromosome 18 band q21, subsequently called *bcl-2* and 18q21, were kindly supplied by Drs C.M. Croce and S.J. Korsmeyer, respectively^{12,13} (Fig 1). The probe 18q21 was a 1.5-kilobases (kb) *Hind*III-*Eco*RI insert. The probe *bcl-2* was a 2.8-kb *Eco*RI-*Hind*III insert. An immunoglobulin heavy chain J_H gene probe was provided by Korsmeyer.²⁰ An *N-myc* probe was obtained from American Type Culture Collection (ATCC, Rockville, MD).

Cytogenetic analysis. Cytogenetic analyses were simultaneously performed on tissue samples from 51 patients using techniques previously described.^{21,22} Direct preparations were performed first and, if insufficient metaphases were observed, short-term cultures were then performed.

RESULTS

Of the 65 samples we analyzed with probes, 31 showed rearrangement of one of the alleles for the 18q21 gene (Table 1). The rearrangements were confirmed by identification of the rearrangement with both probes (Fig 1) in 27 of the 31 cases or by analysis with two or more restriction enzymes (Fig 2A and B). The four exceptions were cases 14, 21, 24, and 28. Cases 14, 21, and 28 were small samples; thus, there was inadequate DNA to repeat the Southern blot analysis. In case 24, the rearrangement could only be detected by using probe 18q21 and enzyme *Pst*-I, with which the analysis was repeated and the result reproduced.

The molecular breakpoints of these rearrangements mostly clustered within the 4.3-kb *Hind*III restriction fragment (Table 1 and Fig 1). Of the 31 cases that showed rearrangement, analysis with *Hind*III restriction enzyme was available in 23; 20 of these 23 (87%) showed rearrangement within the 4.3-kb *Hind*III restriction fragment. The rearrangements in the remaining three cases (cases 2, 29, and 36) were shown by analysis with *Bam*H1, indicating that the breakpoints occurred outside of the 4.3-kb *Hind*III restriction fragment but within the 20.5-kb *Bam*H1 restriction fragment (Fig 1).

Figure 3 is a representative example of comigration of the rearranged 18q21 gene and the immunoglobulin J_H gene. In addition to the 4.3-kb *Hind*III germline fragment, each of the samples had one or two additional rearranged fragments when analyzed with the 18q21 probe (Fig 3, lanes A1, B1, C1, D1, E1, and F1). The 18q21 probe was then removed, and the filters were rehybridized with a J_H probe (Fig 3, lanes A2, B2, C2, D2, E2, and F2). A rearranged fragment of similar size was detected with both the 18q21 (*bcl*-2) probes and the J_H probe in 28 of the 31 samples, suggesting that the 18q21 gene and the J_H gene are on the same fragment. The filters were again washed to remove the J_H probe and rehybridized with a *N-myc* probe as an internal control. All the samples displayed only the expected 16-kb germline fragment,²³ indicating that those rearranged fragments detected with the 18q21 (*bcl*-2) probes and the J_H probe were not the result of technique artifacts of the assay. A summary

of our results correlating molecular genetic analysis with histopathology and karyotypes is shown in Table 2.

Eighteen of 26 (69%) follicular lymphomas (23 FSC/FMs and 3 FLCLs), 3 of 5 (60%) transformed follicular lymphomas, 8 of 20 (40%) DLCLs, 2 of 7 (29%) SNCs, 0 of 5 T cell lymphomas, and 0 of 2 diffuse small lymphocytic lymphomas showed rearrangement of the 18q21 gene (Table 2).

The clinical manifestations and the Ann Arbor clinical stage of the 20 patients with DLCL were reviewed. Comparison of the eight patients who showed rearrangement with the 12 patients who showed no rearrangement revealed differences that are summarized in Table 3. The patients with rearrangement of the 18q21 gene showed a significantly higher frequency of extranodal disease ($P = .03$).

Cytogenetic analysis was attempted on samples from 51 patients (Table 2). In 18 cases, no cells in metaphase were obtained. Twelve of these 18 (67%) showed rearrangement of the 18q21 gene and comigration with the J_H gene, suggesting the t(14;18)(q32;q21) translocation. Of the remaining 33 cases, 13 displayed the t(14;18)(q32;q21) translocation; 10 of these 13 (77%) showed rearrangement of the 18q21 gene. The three cases that showed no rearrangement indicate that the breakpoints in chromosome 18 occurred outside the 20.5-kb *Bam*H1 restriction fragment within which our analysis was confined. In 4 of the 9 cases (44%) that karyotypically displayed a 14q+ chromosome of unknown origin, we could demonstrate a comigrating fragment that hybridized to both the 18q21 (*bcl*-2) probe and the J_H probe, suggesting that a t(14;18)(q32;q21) translocation existed. Three of the 11 with cytogenetic abnormalities other than the t(14;18) or a 14q+ chromosome showed rearrangement of the 18q21 gene (cases 40, 54, and 55). In none of these three cases could we demonstrate a comigrating J_H fragment. The karyotypes of these patients have been carefully reviewed, with emphasis on the structure of chromosome 18. No abnormalities in chromosome 18 could be detected in any of these three.

DISCUSSION

The t(14;18)(q32;q21) chromosomal translocation has been observed frequently in follicular lymphomas but less

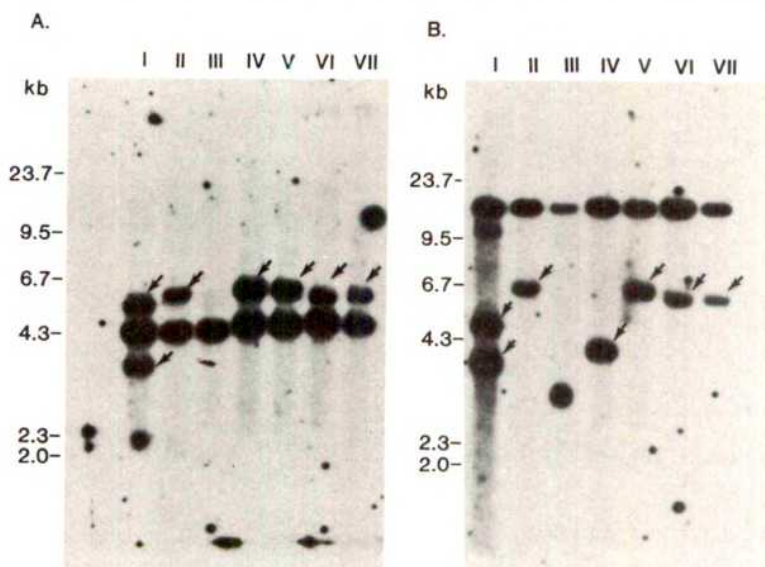


Fig 2. Southern blot hybridization using probe 18q21. The genomic DNAs were extracted from cases 27, 19, 44, 18, 22, 34, and 5, designated I, II, III, IV, V, VI, and VII, respectively. (A) Using enzyme *Hind*III, the 18q21 probe detected a germline band of 4.3 kb. One or two rearranged bands (arrows) were detected in these samples except for case 44 (lane III). (B) Using enzyme *Pst*-I, rearrangements were also demonstrated in cases 27, 19, 18, 22, 34, and 5 (lanes I, II, IV, V, VI, and VII). The sizes of the rearranged bands were similar but not identical.

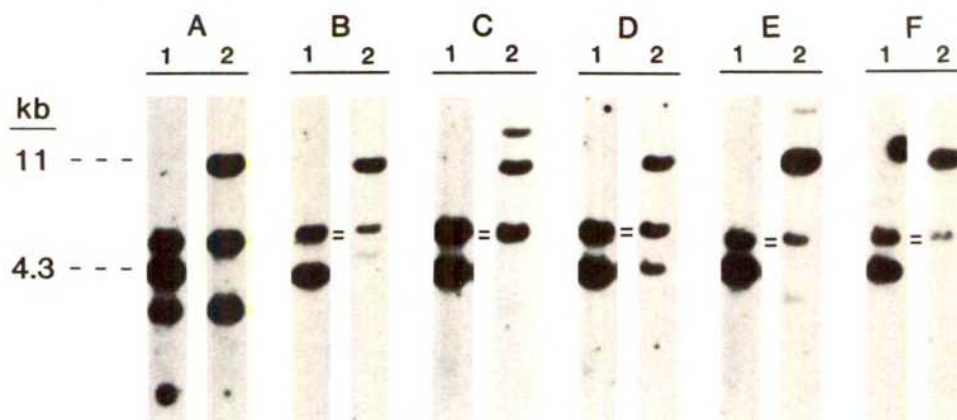


Fig 3. Comigration of the 18q21 gene and immunoglobulin heavy chain J_H gene. The blot shown in Fig 2A was washed and rehybridized with a J_H probe which recognized a 11-kb germline fragment. Autoradiography shown by probe 18q21 (1); autoradiography shown by probe J_H (2). Cases 27, 19, 18, 22, 34, and 5 are designated A, B, C, D, E, and F, respectively. Rearranged 18q21 gene fragments are demonstrated to comigrate with the rearranged immunoglobulin J_H gene fragments (=).

frequently in DLCL.¹⁻³ Several previous studies showed that this translocation results in the rearrangement of putative oncogene *bcl-2*, which resides at 18q21.¹³⁻¹⁵

The t(14;18) is a unique chromosome translocation at the molecular level. Molecular cloning and DNA sequencing of the crossover sites in seven representative cases revealed that breakpoints on chromosome 18q21 were located within 450 base pairs (bp) of each other, and breakpoints on chromosome 14q32 were located close to the 5' end of one of the J segments of the immunoglobulin heavy-chain gene.^{12-14,24-26} The hybrid 18q21- J_H gene is in head-to-tail juxtaposition. The 18q21 gene component is 5' to the J_H gene component. The t(14;18) breakpoint hot spot on chromosome 18q21 is ~2.7 kb 3' to the *Hind*III restriction enzyme site (Fig 1).²⁴⁻²⁶ The breakpoints on chromosome 14q32 usually occur within J4-J6 segment, which is ~2 to 3 kb 5' to the *Hind*III restriction enzyme site.^{12-14,24-26} Therefore, the rearranged bands detected by the 18q21 probe are usually very similar in size (5 to 6 kb) using enzyme *Hind*III. In most cases, two rearranged bands are detected by the *bcl-2* probe since the breakpoints usually fall within this probe.

DNA restriction fragment length polymorphisms are com-

mon problems encountered in the interpretation of gene rearrangement. In our series, most cases with rearrangement were confirmed by at least two restriction enzyme analyses. When the amount of DNA in the samples was small, only one enzyme assay could be performed. The possibility of rearrangement due to DNA polymorphisms is remote because a survey of samples obtained from >20 normal subjects and patients with tumors other than lymphomas revealed no restriction enzyme (*Bam*H1, *Hind*III, *Sst*-1, *Pst*-1) polymorphism related to the 18q21 gene.^{12,13} In occasional cases (cases 14, 21, and 45), the rearrangement was detected by probe *bcl-2* but not by probe 18q21 using restriction enzyme *Hind*III. In these instances, the germline bands detected by the 18q21 probe actually were broader than that of the control sample, suggesting the presence of a rearranged DNA fragment whose size was similar to the germline DNA fragment. The most likely reason for detection of the rearrangement by the *bcl-2* probe is that the breakpoints on chromosome 18q21 in these instances occurred within the *bcl-2* probe that detected "two" rearranged bands, one similar in size to the germline band and the other different in size from the germline band.

We analyzed a 20.5-kb *Bam*H1 segment of this gene in various types of uncultured human lymphomas and demon-

Table 2. Correlation of Histology and Karyotype With Rearrangement of the 18q21 Gene

Histology	Karyotype					Total
	t(14;18)	14q+	NM	Others	ND	
FSC/FM	7/9	1/3	8/9	0/0	1/2	17/23
FLCL	1/1	0/0	0/0	0/0	0/2	1/3
Transf. FL	0/0	0/0	2/2	0/0	1/3	3/5
DLCL	2/2	3/6	2/4	1/5	0/3	8/20
SNC	0/1	0/0	0/0	2/4	0/2	2/7
Others	0/0	0/0	0/3	0/2	0/2	0/7
Total	10/13	4/9	12/18	3/11	2/14	31/65

Numerator, number positive for rearrangement; denominator, number analyzed.

Abbreviations: NM, no metaphases; ND, not done; FSC, follicular small cleaved cell lymphoma; FM, follicular mixed small cleaved and large cell lymphoma; FLCL, follicular large cell lymphoma; Transf. FL, transformed follicular lymphoma; DLCL, diffuse large cell lymphoma; SNC, small noncleaved cell lymphoma.

Table 3. Clinical Stages of 20 Patients With Diffuse Large Cell Lymphoma: Comparison of 8 Cases With Rearrangement and 12 Cases Without Rearrangement of the 18q21 Gene*

Configuration of 18q21 Gene	Stage I-III		Stage IV	
	Nodal Disease	Extranodal Disease†	Bone Marrow Involved	Bone Marrow† Spared
Rearrangement	0	4	0	4
Germline	6	1	4	1

One case showed divergent histology (DLCL in mesenteric lymph node and FM in spleen). The sample that we analyzed displayed DLCL.

*Extranodal disease (including stage IV cases) v nodal disease ($P = .03$ by chi-square analysis).

†Extranodal sites of involvement: base of tongue, palate, thyroid, soft tissue, pericardium, lung, and liver.

strated that a rearrangement within this gene fragment frequently occurs in various types of human B cell lymphomas: 69% of follicular lymphomas, 40% of DLCLs, and 29% of SNCs.

The rearrangement of the 18q21 gene in DLCLs raises a question of whether these cases might actually be follicular lymphomas that transformed into DLCLs. A thorough review of the clinical histories and histopathologies of each case convinced us that they were bona fide de novo DLCLs, except for one in which multiple biopsies from various sites revealed divergent histologies, ie, FM in one site and DLCL in another site. The remote possibility still exists, however, that these cases involved asymptomatic low-grade follicular lymphoma undiagnosed for months or years before transformation.

Review of the clinical manifestations and Ann Arbor stage of the 20 patients with DLCL revealed that the clinical stage of the eight cases in which there was rearrangement of the 18q21 gene was significantly different from that of the 12 cases in which there was no rearrangement. The high frequency of extranodal involvement in the cases with rearrangement is interesting in view of the high frequency of stage IV presentations in patients with follicular lymphoma who typically show the t(14;18) translocation. The clinical behavior of these eight patients will have to be followed closely to determine whether it will be different from that of the 12 patients in whom there was no rearrangement.

Morphological transformation to a more aggressive histologic subtype is a part of the natural history of many follicular lymphomas. Important questions with regard to clonality should be raised: (a) Does the original clone with a rearranged 18q21 gene persist, or does a newly transformed clone take over? (b) Does the original clone acquire a second somatic mutation in the process of histologic transformation?

In our series, three of five cases of transformed follicular lymphomas showed rearrangement of the 18q21 gene. This partly answers the first question, because rearrangement of the 18q21 gene can be preserved in some cases. A precise determination of clonality, however, will require longitudinal follow-up of the cases that showed rearrangement prior to transformation.

Coexistence of the t(14;18) and the t(8;14) translocation has been observed in cell lines derived from a patient with acute B cell leukemia²⁷ and a patient with SNC.¹⁴ In our series, two SNC lymphomas displayed a rearranged 18q21 gene and one carried the t(8;14)(q24;q32) translocation, whereas the other carried the t(8;22)(q24;q11) translocation. The patient with the t(8;14) translocation also had a rearranged *c-myc* oncogene (data not shown). Pegoraro and colleagues postulated that activation of the rearranged 18q21 gene results in a clonal expansion of low-grade malignant B cells, and acquisition of a secondary mutation [eg, t(8;14) or t(8;22)] results in the activation of another oncogene (eg, *c-myc*), leading to a higher grade malignancy.²⁷ To

determine whether these two somatic mutations are sequential events will also require longitudinal studies.

Correlation with cytogenetic analysis in our 13 patients with the t(14;18)(q32;q21) revealed that we could also identify this abnormality by molecular genetic analysis in 10 (77%). That in three cases we were not able to detect the rearrangement suggests that the breakpoints fell outside the 20.5-kb *Bam*HI restriction fragment.

Even though the methodology used in cytogenetic analysis has improved, significant difficulties remain. The technique is cumbersome and requires viable cells. Tumors with a very low proliferative index, such as follicular lymphomas, frequently yield few or no metaphases. Sometimes the donor chromosome for the 14q+ abnormality is uncertain. Furthermore, submicroscopic internal gene rearrangements, if they exist, are impossible to detect by cytogenetic techniques.

Molecular genetic analysis proved very useful in difficult karyotyping situations. We could identify the donor chromosome band as 18q21 in four of nine cases that cytogenetically displayed a 14q+ chromosome of unknown origin. We could also detect the t(14;18)(q32;q21) translocation in 12 of 18 cases that cytogenetically yielded no metaphases. Molecular-genetic analysis has an advantage in that it can be done in tissues stored for months at -20 to -70°C, whereas viable cells are required for cytogenetic analysis. Furthermore, Southern blot analysis requires a small amount of DNA. We were able to perform the molecular genetic analysis simply from the materials of lymph node aspirates. The relatively high frequency (18 of 51) of insufficient metaphases yielded in our studies was in part due to the fact that many of our samples were obtained from lymph node aspirates.

An intriguing finding was the demonstration of three cases (40, 54, and 55) that showed rearrangement of the 18q21 gene without comigration of the *J_H* gene. Further analysis with a *C_μ*, *S_μ*, and *V_H* probe is required to rule out the possibility that the breakpoint on chromosome 14 occurred outside the *J_H* region. The karyotypes in each of these three cases, however, showed an intact chromosome 18, suggesting a submicroscopic rearrangement of the 18q21 gene. Further elucidation of the possible mechanism will require molecular cloning and DNA sequencing.

In summary, rearrangement of the 18q21 gene occurs in various types of B cell lymphomas, including DLCLs and SNCs. For an understanding of clonal evolution, rearrangement needs to be studied longitudinally in transformed lymphomas. Molecular genetic analysis is useful, especially when karyotyping is difficult, and is potentially useful for identifying subsets of lymphoma with unique biological and clinical behaviors.

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Rearranged Antigen Receptor Genes in Hodgkin's Disease

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Despite intensive efforts using a wide variety of approaches, the cellular lineage and clonality of the abnormal cells of Hodgkin's disease have remained an enigma. In the present study, cell separation techniques that enriched for Reed-Sternberg cells and their variants were used to generate sufficient percentages of abnormal cells to allow detection of rearrangements in these cell fractions. DNA from the involved tissues of eight Hodgkin's disease patients was subjected to Southern blot analysis to detect rearrangements of T cell antigen receptor genes and immunoglobulin genes. Immunoglobulin gene rearrangements were found in three of five cases in which Reed-Sternberg cells and their variants were enriched by cell separation techniques to cell frequencies >1%. Rearrange-

ments of immunoglobulin heavy chain genes occurred in two cases, and a λ light chain gene rearrangement occurred in a third case. Rearrangements were not detected in lymphocyte fractions or in unseparated cells prepared from the same tissues. The putative Hodgkin's cell line, L428, also contained rearrangements of immunoglobulin heavy and κ and λ light chain genes and, in addition, harbored a single T cell receptor β gene rearrangement. These findings indicate that Reed-Sternberg cell-enriched fractions contain clonal cell populations and provide a lead, at the molecular genetic level, to a possible lymphoid derivation of the Reed-Sternberg cell.

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THE APPLICATION of monoclonal antibody phenotyping analysis, cytogenetics, and molecular genetics has identified virtually all non-Hodgkin's lymphomas as clonal neoplasms of lymphocytes.¹⁻⁷ In contrast, little is known regarding the cellular derivation, or even clonality, of the Reed-Sternberg cells of Hodgkin's disease (reviewed in refs. 8 and 9). Immunological and cytochemical analyses of Hodgkin's disease tissues have fostered an array of conflicting findings that have led to many alternative speculations. On the basis of these investigations, Hodgkin's disease has variously been suggested to be a neoplasm of B cells,¹⁰⁻¹⁴ T cells,^{14,15} monocytes/macrophages,^{16,17} interdigitating reticulum cells,^{18,19} or myeloid cells.²⁰ Consequently, no definitive features have emerged to classify precisely the lineage of Reed-Sternberg cells.

Cell biology investigations of Hodgkin's disease have been hampered by several natural factors. One prominent obstacle has been the relative paucity of Reed-Sternberg cells in Hodgkin's disease and a vast predominance of supposedly "normal" cellular elements—lymphocytes, plasma cells, histiocytes, and eosinophils.^{8,21,22} Thus, unlike most non-Hodgkin's lymphomas, the presumed neoplastic cells of Hodgkin's disease are a minor fraction of the total cell number, often <1%, which is generally insufficient for functional studies, cytogenetics, or molecular genetic analysis.^{7,23}

Moreover, it has been remarkably difficult to generate and maintain bona fide, continuous Reed-Sternberg cell lines. Although several cell lines have been cultured, some of these either have not been immortalized²⁴ or were contaminated by

cells derived from other sources.²⁵ One cell line, L428, derived by Diehl and colleagues from the pleural effusion of a patient with previously treated nodular sclerosing Hodgkin's disease has been maintained for >6 years.²⁶ Whether the L428 cell is indeed the direct descendant of a Reed-Sternberg cell, a secondary neoplasm, or a lymphoblastoid cell is not known, since the primary Reed-Sternberg cells from that patient have not been genetically compared with the L428 cell line. The L428 cell remains a possible candidate as a Reed-Sternberg cell, however, since it carries karyotypic abnormalities,²⁶ shares an immunoreactive surface membrane protein with Reed-Sternberg cells (Ki-1 or Hefi-1),^{27,28} and has a phenotype slightly reminiscent of the Reed-Sternberg cell.²⁶

The focus of the present study was to determine the clonality and lineage of Reed-Sternberg cells by molecular genetic analysis of their immunoglobulin and T cell receptor genes. The success of this approach holds promise in that it is sensitive, provides direct evidence of clonality without necessitating cytogenetics, and can be used to identify lineage-related markers without gene expression.^{7,23,29-31} Because cells from Hodgkin's disease might be separable by physical methods,³² our strategy was to apply cell separation procedures to enrich the percentage of Reed-Sternberg cells to threshold levels needed to detect gene rearrangements by Southern blot analysis. By isolating Reed-Sternberg cells, we have begun to explore the molecular biology of Hodgkin's disease.

MATERIALS AND METHODS

Tissues and cells. Involved lymph nodes and spleens were obtained from eight patients with nodular sclerosing Hodgkin's disease (Table 1). Cases were unselected, and no attempt was made to restrict the study to cases with high percentages of Reed-Sternberg cells. Cases of lymphocyte-depleted Hodgkin's disease were excluded from analysis because of the high likelihood that many may actually be non-Hodgkin's lymphomas.^{33,34} Cases of mixed cellularity or lymphocyte-predominant Hodgkin's disease were not available. All patients were advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent.

Fresh tissue samples were finely minced in RPMI 1640 culture medium (GIBCO, Grand Island, NY) and subsequently digested

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with collagenase type IV 1.0 mg/mL (Sigma Chemical, St Louis) for 30 to 90 minutes at room temperature with constant stirring. Cell viabilities throughout all procedures were consistently >90%. Cyto-centrifuge slides of the cell suspensions were prepared for morphological and immunostaining analyses.

Cell suspensions were fractionated by Ficoll-Hypaque (Lymphoprep, Accurate Chemical and Scientific, Westbury, NY) or Percoll (Pharmacia, Piscataway, NJ) density-gradient centrifugation or by elutriation. Ficoll-Hypaque separation was performed by layering 8 mL of suspended cells ($\sim 10^7$ /mL) over 4 mL of Ficoll-Hypaque in a 15-mL conical tube for 30 minutes at 400 g at room temperature. Both the interface and pellets were saved for analysis. Percoll solutions were prepared in accordance with the instructions of the manufacturer using 45% to 60% isosmotic Percoll solutions with a density ranging from 1.050 to 1.076 g/mL. An aliquot of 1 mL of cells at 5×10^7 /mL was spun through 9 mL of gradient for 20 minutes at 400 g at room temperature. A control gradient containing standardized beads was simultaneously run to determine density positions. Morphological analysis of Wright's-stained cyto-centrifuge slides was used to evaluate the optimal density range for separation of Reed-Sternberg cells and variants from the remaining cell population. Once this density range was determined, a two-step discontinuous isosmotic Percoll gradient was performed, which trapped the enriched Reed-Sternberg cell fraction between the selected densities (1.051 and 1.069 g/mL). A large volume of cells (20 to 25 mL at 5×10^7 /mL) was separated using the discontinuous Percoll gradient.

The splenic cells obtained from Hodgkin's patients were also separated by counterflow centrifugal elutriation with a Beckman JE-6B elutriation system and rotor as previously described,³⁵ with the exception that the separation media was Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium (Biofluids, Rockville, MD). Prior to elutriation, 4 to 8×10^8 spleen cells were incubated at 37°C in 40 mL of DPBS containing 1 mg/mL of deoxyribonuclease (Sigma) for 30 minutes. The cells were then loaded into the elutriation rotor, which was spinning at 1,960 rpm, at a flow rate of 8 mL/min. One hundred fifty milliliters were collected at this flow rate and constituted the first fraction. Subsequently, the cells in fractions 2 through 6, each containing 150 mL, were collected at flow rates of 11, 13.6, 16.4, 18.0, and 19.2 mL/min, respectively. The last fraction, number 7, was comprised of cells that eluted at the final flow rate after the centrifuge was stopped.

Samples from appropriate fractions were viably stored over liquid nitrogen as described. Cyto-centrifuge slides were prepared and used for differential counts (1,000 cells) and immunostaining (see below). DNA was extracted from the majority of cells (1 to 3.5×10^8 cells), as outlined below.

Cell lines used in these studies were L428²⁶ (V. Diehl, University of Cologne) and the African Burkitt lymphoma, Raji (American Type Culture Collection, Rockville, MD). Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque gradient from a normal individual.

Immunophenotyping. Cyto-centrifuge preparations were stained with monoclonal antibodies using an indirect avidin-biotin-peroxidase complex (ABC).³⁶ The percentage of positive cells was determined by scoring 500 to 1,000 cells per slide.

Monoclonal antibodies and their sources included Leu-1, Leu-2, Leu-3, Leu-4, and Leu-M1 (Becton Dickinson Monoclonal Antibodies, Sunnyvale, CA); B-1, B-4, (Coulter Immunology, Hialeah, FL); Hefi-1 (Dr Richard Fisher, Loyola University); and anti-Tac (Dr Thomas Waldmann, National Cancer Institute).

Gene rearrangement analysis. High-mol-wt DNA was extracted from unseparated specimens, Reed-Sternberg cell-enriched fractions, Reed-Sternberg cell-depleted fractions, and the L428 cell line²⁶ by a standard phenol-chloroform ethanol precipitation technique.³⁶ When only small numbers of cells ($<40 \times 10^6$) were

available, DNA was extracted by ultracentrifugation with a guanidine thiocyanate-cesium chloride gradient.³⁶ DNA was subjected to restriction fragment digestion, Southern transfer, and probed with genomic clones of human immunoglobulin J_H, J_K, C_κ, C_λ, T cell γ gene J region (J_γ) and cDNA of the T cell receptor β subunit constant region (C_β), and a full-length cDNA (T_α), as detailed in previous reports.³⁷⁻³⁹ For each sample, BamHI-, EcoRI-, and HindIII-digested DNAs were probed with J_H and C_β, BamHI digests with C_κ, and EcoRI digests with C_λ, J_γ, and T_α probes were hybridized with blots containing L428 DNA digested with BamHI, EcoRI, and HindIII.

For Southern blot sensitivity analysis varying proportions of DNA obtained from Raji cells were mixed with DNA from normal polyclonal splenic lymphocytes. DNA was digested with EcoRI, and conventionally prepared Southern blots were probed with J_H.

RESULTS

Cell separation. Unfractionated cell suspensions of tissues involved by Hodgkin's disease contained <1% Reed-Sternberg cells and variants, except case 4, which was comprised of 4% abnormal cells (Table 1). Percentages displayed in Table 1 represent counts determined by morphological analysis of cyto-centrifuge preparations, which were confirmed in all instances by parallel cyto-centrifuge slides stained with Leu-M1,¹⁹ Tac¹⁹ and Hefi-1.²⁸ In each case, most normal cells were lymphocytes that expressed either T or B cell surface markers. Reed-Sternberg cells and their variants were unreactive with all anti-T cell antibodies, B-1, and B-4. In the elutriation cell separation experiments, Reed-Sternberg cells and their variants increased in fractions 5 through 7; thus, these fractions were combined in case 2 with a yield of 6.6% Hodgkin's cells. Enriched fractions of Reed-Sternberg cells were isolated from discontinuous Percoll gradients in cases 1, 4, 5 and 7 (Figs 1 and 2) using predetermined optimal density ranges (Fig 1); the results of large-scale gradient centrifugation separations are listed in Table 1.

Leu-M1 and Hefi-1 staining confirmed the percentages of Reed-Sternberg cells and variants in the enriched fractions (Fig 3). Lymphocyte fractions contained <0.1% Leu-M1- or Hefi-1-positive cells in all cases. As previously demonstrated by other researchers,^{40,41} Reed-Sternberg cells often formed

Table 1. Hodgkin's Disease Cell Separations

Case	Reed-Sternberg Cells (and Variants) (%)		Gene Rearrangements			
	Unseparated	Separated	IgH	Ig _κ	Ig _λ	T _β
1	0.05	0.70*	G	G	G	G
2	0.40	6.60†	G	G	R	G
3	0.05	ND	G	G	G	G
4	4.00	9.00*	G	G	G	G
5	0.04	4.80*	G	G	G	G
6	0.60	9.00‡	RR	G	G	G
7	0.03	1.60*	R	G	G	G
8	0.05	ND	G	G	G	G
L428			R	RG	RG	RG

*Percoll gradient; †, elutriation; ‡, Ficoll-Hypaque gradient.

IgH, Ig_κ, Ig_λ, immunoglobulin heavy, κ and λ chain genes; T_β, T cell receptor β chain gene; G, germline; R, rearranged; RR, two rearrangements; ND, not done (separations not performed due to insufficient cells).

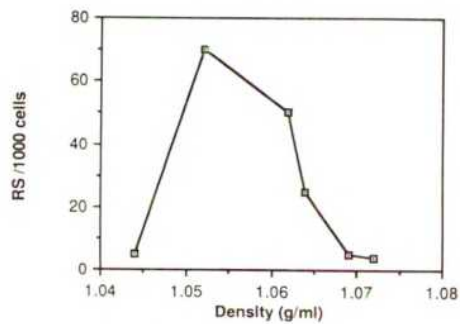


Fig 1. Percoll gradient centrifugation. A cell suspension of a lymph node involved by Hodgkin's disease was centrifuged through a continuous Percoll gradient as described in the Materials and Methods Section. Cell samples were taken from several densities, and the optimal density for enrichment of Reed-Sternberg cells and variants (RS) was determined by morphological analysis of cytocentrifuge preparations. In this example (case 5), peak RS separation was obtained between 1.051 and 1.069 g/mL.

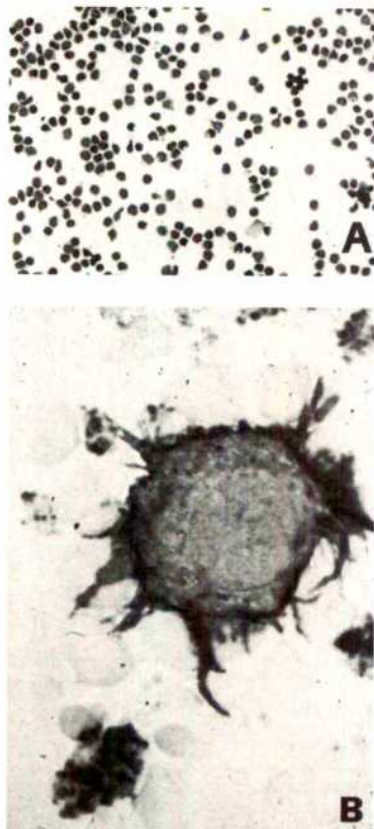


Fig 2. Density gradient separation of Reed-Sternberg cells and variants (case 5). A cell suspension of an involved lymph node, which contained 0.04% Reed-Sternberg cells and variants, was fractionated by centrifugation through a discontinuous Percoll gradient. (A) Lymphocytes (>99%) were recovered from the pellet (density >1.069 g/mL) (Original magnification $\times 400$; current magnification $\times 263$; Wright's stain). (B) Reed-Sternberg cells and their mononuclear and multinuclear variants were highly enriched in the interface at density 1.069 g/mL (original magnification $\times 400$; current magnification $\times 270$, Wright's stain).

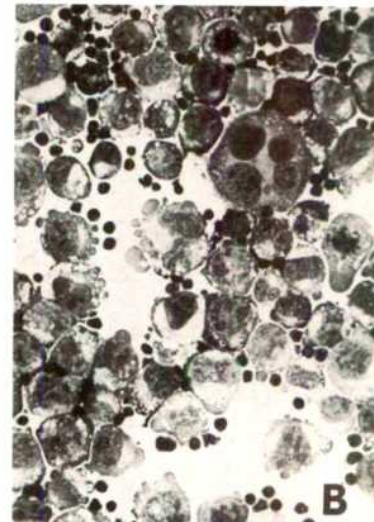


Fig 3. Immunostaining of cytocentrifuge preparations of Reed-Sternberg cell-enriched fraction (case 5). (A) Spontaneous adherence of T cells to mononuclear variants of Reed-Sternberg cells. Reed-Sternberg cells and variants did not react with the anti-T cell antibody Leu-4 (Leu-4, ABC; original magnification $\times 1,000$ current magnification $\times 6,500$). (B) Leu-M1 staining of a mononuclear variant of a Reed-Sternberg cell reveals elaborate dendritic processes that engulf adherent T cells (Leu-M1, ABC; original magnification $\times 1,000$, current magnification $\times 6,500$).

spontaneous rosettes with autologous T cells (Fig 3A). Dendritic processes elaborated by Reed-Sternberg cells and their variants were stained by Leu-M1 and appeared to engulf adjacent T cells (Fig 3B). The functional meaning of this intercellular activity is not known.

Gene rearrangement. DNA obtained from intact, unseparated, Hodgkin's disease samples was initially screened for gene rearrangements. As in previous studies using this approach,³⁰ no rearrangements were detected (Fig 4). Therefore, subsequent analyses were performed with DNA from fractionated cell populations. Sufficient DNA (usually >100 μ g) was extracted from fractions of interest to allow analysis with each probe and multiple restriction enzymes. In three of

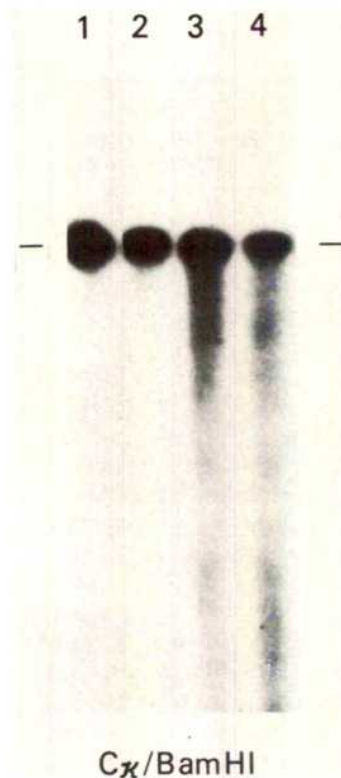


Fig 4. Southern analysis of immunoglobulin κ genes from unfractionated Hodgkin's tissues. Filter hybridization of *Bam*HI digests of genomic DNA obtained from cases 1 through 4 showed no evidence of κ gene rearrangement. Case numbers are shown above each lane. Adjacent dashes indicate the germline *Bam*HI restriction fragment size (12 kilobase, kb) hybridizing with the C_κ probe.

the eight cases (cases 2, 6, and 7), immunoglobulin gene rearrangements were identified in the Reed-Sternberg cell-enriched fractions (Fig 5, Table 1). In contrast, in these same cases, rearrangements were not apparent in either the unseparated cells or in the Reed-Sternberg-depleted (lymphocyte) fractions (Fig 5, Table 1). Thus, detectable gene rearrangements were specifically associated with fractions containing $>1\%$ Reed-Sternberg cells and variants. No T_β gene rearrangements were detected in the cases.

The Reed-Sternberg cell-enriched fraction in case 2 showed one rearranged λ light chain allele, but no rearrangements of immunoglobulin heavy chain, κ light chain, or T_β genes were seen. Although light chain gene rearrangements are found in B cells, it is unusual to find a light chain gene rearrangement without a concomitant heavy chain gene rearrangement.⁴² This finding might be attributable to a technical factor, such as comigration of rearranged and germline bands, but no J_H rearrangements were detected despite digestion with *Bam*HI, *Eco*RI, and *Hind*III. Potentially, deletion of a substantial portion of the J_H region during rearrangement might diminish the sensitivity of detection of J_H hybridization. Simultaneous hybridization with the same labeled probe on the same filter, however, demonstrated rearrangements in cases 6 and 7 (Fig 5, Table 1). Alternatively, the λ gene rearrangement in case 2 may have resulted

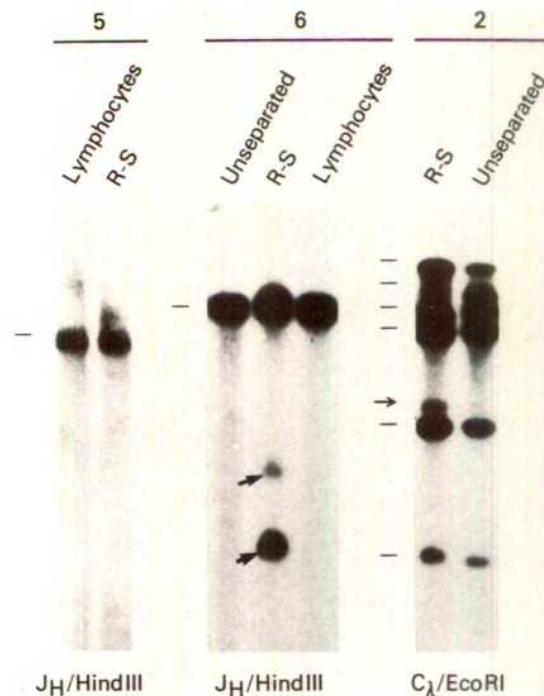


Fig 5. Immunoglobulin gene rearrangements in Reed-Sternberg cell-enriched fractions. Cell types were separated as outlined in Table 1, and DNA was extracted and analyzed for gene rearrangement in Southern blots. Case numbers are at top. In case 5, no rearrangements of the immunoglobulin heavy chain gene were detected in either the lymphocyte or Reed-Sternberg cell-enriched (RS) fractions. The RS fraction from case 6 contained two nongermline bands (arrows) hybridizing with J_H in *Hind*III-digested DNA. In contrast, no rearrangements were seen in either the unseparated cells or lymphocyte fractions from case 6. In case 2, the RS fraction exhibited a single rearrangement of the λ gene (arrow), whereas no λ rearrangements were detectable in unseparated cells; germline sizes (-).

from chromosomal breakage or DNA deletion within a hybridizing restriction fragment rather than variable region gene joining. No cytogenetics were available, and sequence analysis would be required to resolve this point.

Cases 6 and 7 both displayed J_H rearrangements and no light chain or T_β gene rearrangements. Again, nondetection of rearrangements might be due to differential sensitivities of each probe. Because heavy chain genes are known to rearrange prior to light chain genes in pre-B cells,⁴² our findings may imply a pre-B cell stage of development. This cannot be concluded with certainty, however, since one cannot reliably exclude the possible existence of a light chain gene rearrangement in such a minor cell population.

L428 cells were found to contain gene rearrangements that included one heavy chain, one κ light chain, one λ light chain, and one T_β chain gene (Fig 6). Restriction fragment analysis of the T_α and J_γ genes showed only germline hybridizing bands.

To estimate the sensitivity of Southern blot analysis for the detection of rearrangements in rare clones, a dilution experiment was performed. The B cell (Burkitt's) lymphoma line, Raji, carries two rearranged J_H genes as seen in *Eco*RI-digested DNA. When known amounts of Raji DNA were

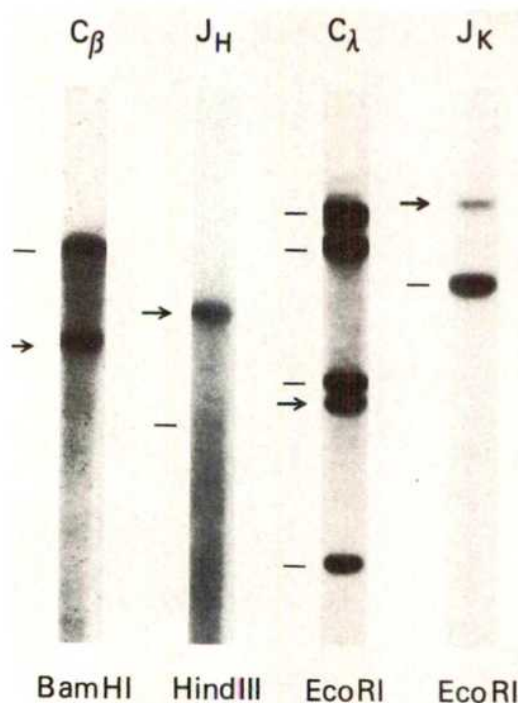
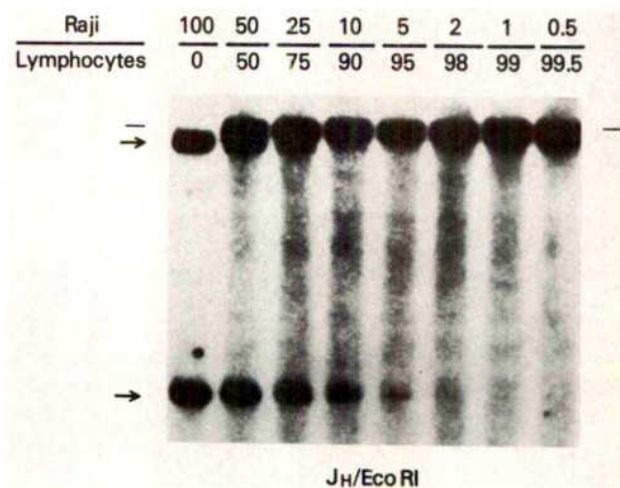


Fig 6. Gene rearrangements in the L428 Hodgkin's cell line. By Southern analysis, single gene rearrangements were detected for the β subunit of the T cell antigen receptor (C_β), immunoglobulin heavy chain (J_H), κ light chain (J_κ), and λ light chain (C_λ) (arrows). Location of germline restriction fragments (-).

mixed with DNA obtained from normal polyclonal lymphocytes and digested with *EcoRI*, nongermline bands contributed by Raji cells could still be detected when Raji DNA was diluted to only 2% of the total DNA (Fig 7). Therefore, clones with a frequency of as little as 2% of the cell population are detectable by Southern analysis, thus confirming the findings in Reed-Sternberg cell-enriched fractions.

DISCUSSION

By enriching for Reed-Sternberg cells and their variants using cell separation techniques, we uncovered immuno-



globulin gene rearrangements in Hodgkin's disease. In each of the three primary cases in which rearrangements were detected, Reed-Sternberg cells and variants were enriched to account for >1% of the cell population, a level that was shown to be near the threshold of sensitivity for Southern analysis. Rearrangements were not observed in any of the samples containing <1% Reed-Sternberg cells and variants. Thus, lack of detection of rearrangements in Hodgkin's disease tissue may be a consequence of subthreshold percentages of Reed-Sternberg cells. It should be emphasized that cases studied here were unselected, yet were unequivocally diagnostic examples of Hodgkin's disease. No attempt was made to examine tissues with high percentages of Reed-Sternberg cells specifically, a procedure that might permit inadvertent inclusion of some non-Hodgkin's large cell lymphomas due to difficulties inherent in differential diagnosis.^{33,34} Furthermore, analyses were performed on lymph nodes and spleens involved by Hodgkin's disease, in which the diagnosis is most accurately made.⁴³ With this approach, we avoided ambiguities regarding circulating neoplastic cells⁴⁴ that might well be secondary non-Hodgkin's lymphoma or leukemia arising in a patient with Hodgkin's disease. Based on the findings reported here, evidence that rearrangements occurred in the Reed-Sternberg cells per se is circumstantial, since cell fractions were not purified to homogeneity. For example, although unlikely, a minor clonal B cell or plasma cell population with a density or volume comparable to Reed-Sternberg cells may have been concomitantly enriched in the Reed-Sternberg-containing fractions. Documentation of the gene rearrangements of Reed-Sternberg cells will necessitate the elimination of extraneous cells by further cell purifications. An additional finding of the present study is that no gene rearrangements were identified in the lymphocyte fractions of lymph nodes containing Hodgkin's disease. Thus, no significant clonal population was contained among these cells. Furthermore, the lymphocyte fraction served as a germline control for each patient to guard against restriction fragment length polymorphism.

Our findings indicate a heterogeneity of rearranged genes among cases of Hodgkin's disease. Rearrangement of immunoglobulin heavy but not light chain genes occurred in two cases (6 and 7), and the converse was seen in case 2. Two

Fig 7. Sensitivity of Southern blot. Raji DNA was diluted in DNA obtained from normal polyclonal splenic lymphocytes. The percentages of total DNA contributed by either Raji or lymphocytes is indicated above each lane. DNA was digested with *EcoRI* and probed with J_H . Germline fragment (-); the two rearranged J_H loci in Raji cells (arrows).

cases (4 and 5) with fractions containing relatively abundant Reed-Sternberg cells had no demonstrable rearrangements for immunoglobulin or T cell receptor β chain genes. The inconsistent rearrangement pattern from case to case may simply be technical; thus, rearrangements in minor cell populations might remain undetected. Alternatively, rearrangements may not have occurred, perhaps as a result of incomplete attempts at B cell differentiation. Cells might be arrested at a pre-B cell stage in which immunoglobulin heavy but not light chains rearrange.⁴² Pre-B cells, however, contain terminal deoxynucleotidyl transferase, and this enzyme is not seen in Hodgkin's disease.⁴⁵ Rearrangements resulting from chromosomal breaks rather than immunoglobulin variable region gene recombination could give rise to the Southern blot pattern we observed. Whether chromosomal damage might account for rearrangements in the present cases is not known since cytogenetics were not performed, but some reported examples of Hodgkin's disease carry abnormalities of the long arm of chromosome 14,⁴⁶ where the immunoglobulin heavy chain gene is located.^{47,48}

By identifying immunoglobulin gene rearrangements, our molecular genetic analysis of the Reed-Sternberg-enriched cell fractions demonstrated minor clonal cell populations in Hodgkin's disease. This finding, coupled with earlier reports of clonal chromosomal abnormalities,⁴⁶ argues that Reed-Sternberg cells are clonally expanded in Hodgkin's disease. Whether the clonal cells detected by either approach are indeed the Reed-Sternberg cells or some other cellular component of Hodgkin's disease associated with Reed-Sternberg cells remains to be determined, however.

Our study of Reed-Sternberg cell-enriched fractions corroborates the demonstration by Weiss and associates⁴⁹ of immunoglobulin gene rearrangements in cases of Hodgkin's disease with abundant Reed-Sternberg cells. In that study, as in the present report, rearranged heavy chain genes were found without accompanying light chain gene rearrangements in some cases, a feature that deserves further investigation. In another study, rearranged T_{β} genes in four cases but no immunoglobulin gene rearrangements were reported from a series of eight cases of Hodgkin's disease.⁵⁰ In that study, however, T_{β} rearrangements were detected with a probe contaminated with variable and joining gene sequences that could produce numerous additional germline bands, which are difficult to interpret without matched germline control tissue from the same patient.⁵¹ Rearrangements of either T_{β} or immunoglobulin genes were also identified by Knowles and colleagues in 3 of 15 cases of Hodgkin's disease, but it was concluded that rearrangements were attributable to clonal lymphoid cells rather than Reed-Sternberg cells.⁵¹ No direct evidence was provided, however, to substantiate such an interpretation. As shown in the present report, lack of rearrangements as observed by others would be expected

in a study of unseparated samples. Furthermore, the actual percentage of Reed-Sternberg cells in a specimen is extremely low and probably less than would appear by estimation from a single histological section. Thus, without direct cell counting of the sample, cases with apparently "numerous" Reed-Sternberg cells might well contain an insufficient percentage of abnormal cells for detection of rearrangements by Southern blot. Finally, when a differential diagnosis of Hodgkin's v non-Hodgkin's lymphoma must be made, it is premature at present to derive a diagnosis of Hodgkin's disease based on the *absence* of rearrangement as suggested by Knowles and colleagues.⁵¹ This precaution should be seriously considered in light of the issues that affect the interpretation of gene rearrangements in a rare cell subpopulation, as well as findings reported here and elsewhere.⁴⁹

Rearrangements of immunoglobulin heavy and κ and λ light chain genes in the L428 cell line suggest a B cell lineage for this cell. The usual hierarchy of light chain gene rearrangement⁴² has been violated, since κ light chain gene analysis demonstrated a single band of germline configuration. Thus, one of the two κ alleles has been neither rearranged nor deleted, as would be expected in a B cell with a rearranged λ gene. An explanation for this unexpected rearrangement of light chain genes might be that, since the C_{λ} locus is polymorphic, the single nongermline C_{λ} hybridizing band obtained with *EcoRI* is a restriction fragment length polymorphism. The size of the *EcoRI* fragment (7.0 kilobase, kb) however, is not the size of any of the known polymorphic *EcoRI* fragments (8, 13, 18, and 23 kb)⁵² and therefore can be considered a consequence of rearrangement of the λ gene locus. In addition, L428 carries one rearranged T_{β} gene, an occasional feature of B cell neoplasms.^{53,54} T_{β} gene rearrangements and germline immunoglobulin genes were found in a T cell line, CO, derived from a patient with Hodgkin's disease, but it is not known whether this cell line represents the tumor.⁵⁵ The unusual molecular genetic characteristics of L428 elude precise classification of cellular lineage, but its rearrangement of immunoglobulin genes is a feature shared with our primary cases of Hodgkin's disease; together, these findings might provide a clue as to the derivation of the abnormal cells of Hodgkin's disease.

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Expression on Blood Cells of Sialophorin, the Surface Glycoprotein That Is Defective in Wiskott-Aldrich Syndrome

By Eileen Remold-O'Donnell, Christopher Zimmerman, Dianne Kenney, and Fred S. Rosen

Sialophorin, previously called gpL115, is the heavily sialylated surface protein that is defective in lymphocytes of Wiskott-Aldrich syndrome patients. Using the monoclonal antibody L10 as a probe, sialophorin expression was detected on isolated T lymphocytes and thymocytes, B cell lines, monocytes, neutrophils, and platelets, but not on erythrocytes, fibroblasts, and glioblastoma cells. This unusual distribution pattern suggests that sialophorin is expressed on all circulating cells except erythrocytes. Trace amounts of the sialophorin molecules on lymphocytes are incompletely sialylated, but significant amounts of the molecules on thymocytes are incompletely sialylated. The molecular form of sialophorin on T lymphocytes, thymocytes, and monocytes is the previously characterized species of apparent mol wt 115,000. A newly described

sialophorin species of apparent mol wt 135,000 was found on neutrophils and platelets. The 115,000 lymphocyte/monocyte form and the 135,000 platelet/neutrophil form were shown to be substantially similar. The two forms have approximately the same content of sialylated O-linked carbohydrate units since both undergo the same atypical shift in electrophoretic mobility on desialylation. Both contain the epitope recognized by the monoclonal antibody L2 and the epitope recognized by L10 antibody. Moreover, evidence from another study indicates that the polypeptide portions are identical, cumulatively suggesting that 115,000 sialophorin and 135,000 sialophorin are identical except for the presence on the latter of additional neutral saccharide residues.

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SIALOPHORIN, previously called gpL115, is the heavily glycosylated lymphocyte surface protein that is altered in the X-linked disorder Wiskott-Aldrich syndrome (WAS).^{1,2} WAS patients have severe dysfunction of platelets (reduced platelet size, number, and function) and of lymphocytes.^{3,4} The lymphocyte functional defect is reflected in recurrent infections, eczema, decreased antibody production to carbohydrate antigens, and decreased proliferative responses in vitro to mitogens and antigens.

In earlier studies, we compared ¹²⁵I-labeled lymphocytes of WAS patients and normal individuals.^{1,2} Sialophorin (gpL115), a surface glycoprotein of apparent mol wt 115,000, was undetectable, or reduced in quantity, and/or present in an abnormal form in eight WAS patients.² Sialophorin identical to the molecule on normal lymphocytes² was purified from the lymphoblastoid line CEM and shown to consist of a ~520 amino acid polypeptide rich in serine, threonine, and proline, ~two N-linked carbohydrate units, and ~100 O-linked units of the sialylated disaccharide galactose-N-acetylgalactosamine.^{5,6}

As a step toward establishing the physiological role of sialophorin and understanding its dysfunction in the WAS, we examined the expression of the molecule on isolated blood cells. We report that there are two forms of sialophorin on blood cells and characterize the distribution of the two species.

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MATERIALS AND METHODS

Blood fractionation. Fresh acid-citrate-dextrose, NIH formula A, (ACD)-anticoagulated blood was centrifuged at 2,500 rpm for 3 minutes in a Sorvall HG rotor. Fractions were collected from the blood bag under pressure as follows: platelet-rich plasma (PRP), buffy cells (60 to 80 mL), and erythrocytes (lower half of packed cells); these were processed at room temperature. Hanks' balanced salt solution (HBSS) and media contained 100 U/mL of penicillin and 100 µg/mL of streptomycin.

Lymphocytes. Buffy coat cells were diluted with equal volume Ca²⁺/Mg²⁺-free HBSS with 20 µg/mL of leupeptin, and 20-mL portions were layered onto 17 mL of Ficoll/Hypaque. After centrifugation, the interface mononuclear cells were washed 1 to 4 times in Ca²⁺/Mg²⁺-free HBSS with 2% fetal calf serum (FCS) to remove contaminating platelets. Washed MNLs at 5 × 10⁶/mL in RPMI 1640 with 2% FCS were cultured for 1 hour at 37°C in culture flasks. The nonadherent cells were collected.

T lymphocytes. Lymphocytes were allowed to react with sheep RBCs (SRBCs).⁷ Rosetted cells were pelleted by Ficoll-Hypaque centrifugation, subjected to hypotonic lysis to remove erythrocytes, and pelleted to yield cells that were 97% CD3-positive as detected by fluorescein-conjugated Leu-4 (Becton Dickinson, Mountain View, CA).

Monocytes. Tissue culture dishes (60 × 15 mm) were pretreated with 2 mL of FCS at 37°C for 15 minutes. After removal of the FCS, 15 × 10⁶ mononuclear cells in 3 mL of RPMI with 10% FCS were cultured for 60 minutes at 37°C. Nonadherent cells were aspirated; the monolayer was washed with RPMI with 2% FCS at 37°C. Adherent cells were harvested with a pipette and a rubber policeman after cooling to 4°C. The collected cells were ≥90% peroxidase-positive mononuclear cells; monocyte recovery was ≥90%. In some experiments, monocytes were cultured for 7 days in RPMI with 10% FCS as adherent cells or as nonadherent cells at 2 × 10⁶/mL in Teflon beakers (Nalge Co, Rochester, NY).

Neutrophils. Pelleted blood cells (after removal of PRP) were combined with equal volume 2% dextran in 0.15 mol/L of NaCl for 30 to 40 minutes at 22 ± 2°C to sediment erythrocytes. The supernatant was aspirated, and the leukocytes were fractionated on Ficoll-Hypaque. The pelleted cells were washed in Ca²⁺/Mg²⁺-free HBSS, and residual erythrocytes were removed by water lysis. The cells were 96% neutrophils (Wright's stain).

Platelets. PRP was made 5 mmol/L in EDTA and was combined with 0.2 vol ACD and centrifuged at 800 g × 15 minutes. The pelleted platelets were resuspended in N-Tris-(hydroxymethyl) methyl-2-aminoethane sulphonic acid (TES)/Tyrodes' buffer (10

mmol/L-TES, 136 mmol/L of NaCl, 2.6 mmol/L of KCl, 0.5 mmol/L of NaH_2PO_4 , 2 mmol/L of MgCl_2 , 5.5 mmol/L of glucose, pH 7.4) containing 5 mmol/L of EDTA and washed twice by centrifugation at 800×12 minutes. Platelet preparations contained <1 WBC per 4,000 platelets (Coulter Model S Plus IV).

Thymocytes. Normal thymocytes, teased from thymic tissue removed as a routine part of open heart surgery, were filtered through sterile cotton gauze and were pelleted. (Thymocytes further purified on a Ficoll-Hypaque gave comparable results.)

Cell lines. The human T lymphoblastoid cell lines CEM^{5,8} and MOLT-4⁹ (ATCC) were grown in Dulbecco's medium with 4.5 mg/mL of glucose and 10% FCS. Cell lines were cultured with 100 U/mL of penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin. The T lymphoblastoid cell lines HPB-MLT, HPB-ALL, JURKAT, and HUT-78¹⁰ and the B cell line JY (donated by Dr Steven Mentzer, Dana-Farber Cancer Institute, Boston) were grown in RPMI 1640 with 10% FCS. A172, human glioblastoma cells¹¹ and MRC-5 cells,¹² normal human fibroblasts from fetal lung tissue (ATCC) were grown as adherent cells in culture flasks in Dulbecco's medium with 4.5 mg/mL of glucose and 10% FCS and treated at confluence with 0.5% trypsin-0.02% EDTA (Flow Laboratories, McLean, VA).

Diisopropylfluorophosphate and sialidase treatment and lysis of cells. Nucleated cells washed twice at 4°C were incubated with 4 mmol/L of diisopropylfluorophosphate (DFP) without or with 0.02 IU/mL of *Vibrio cholerae* sialidase as described,² diluted with cold HBSS, and pelleted. Nucleated cells (2.5×10^7 or 5×10^7 thymocytes) were extracted with 1 mL of 0.5% NP-40, 10 mmol/L of Tris-HCl, pH 7.4, 150 mmol/L of NaCl, 1 mmol/L of DFP, 3 mmol/L of iodoacetamide for 3 minutes at $22 \pm 2^\circ\text{C}$ and 8 minutes at 4°C . Insoluble material was removed at 12,000 g for 13 minutes. MRC-5 cells were lysed at passage number ~ 35 . Platelets at $2 \times 10^9/\text{mL}$ in TES/Tyroses' buffer with 5 mmol/L of EDTA were solubilized with 1 vol 2% sodium dodecyl sulfate (SDS), 1% NP-40, 20 mmol/L of Tris-HCl, pH 7.4, 150 mmol/L of NaCl at 100°C for 1 minute. Erythrocytes washed three times in cold phosphate-buffered saline (PBS) were lysed in 5 mmol/L of sodium phosphate buffer, pH 8.0 at 4°C . The "ghosts" were washed free of hemoglobin by pelleting¹³ and solubilized at 2 mg protein/mL in 1% SDS, 0.5% NP-40, 10 mmol/L of Tris-HCl, pH 7.4, 150 mmol/L of NaCl, 1 mmol/L of DFP, and 3 mmol/L of iodoacetamide. Some cell extracts were treated with *Vibrio cholerae* sialidase as described.²

Monoclonal antibodies. L10 is a mouse IgG₁ that binds to a cell surface epitope on native and asialo-sialophorin.^{2,5} L2, an anti-sialophorin antibody from a parallel fusion, is an IgE. In double diffusion, L2 reacts with rabbit anti-mouse IgE antiserum giving a line of identity with pure IgE (reagents supplied by Drs Alfred Nisonoff and Seiji Haba, Brandeis University, Waltham, MA) and shows no reaction with isotype-specific rabbit antisera to mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgM, and IgA (Litton Bionetics, Charleston, SC). ³⁵S-labeled L2 antibody, obtained by culturing L2 cells with [³⁵S]methionine, was immunoprecipitated by rabbit anti-mouse IgE antiserum; its apparent mol wt is $\sim 250,000$ (nonreduced) and 92,000 and 26,000 (reduced). L2 antibody was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE cellulose chromatography. ¹²⁵I-labeled L2 bound to 260,000 sites per native CEM cell and L10 to 190,000 sites (three experiments; method and L10 data in ref. 5). L2, unlike L10, does not bind to sialidase-treated cells. L2 immunoprecipitates native but not asialo-sialophorin.

SDS electrophoresis and immunoblotting. Cell extracts were heated with equal volumes of 2% SDS in 60 mmol/L of Tris-glycine buffer, pH 6.8 for 2 minutes at 100°C and electrophoresed on 1.5-mm 7.5% polyacrylamide gels.² Near quantitative transfer of sialophorin to 0.2- μm nitrocellulose sheets¹⁴ (Schleicher and Schuell, Keene, NH) was achieved by constant 2.1 A (250 V/2.5 A power supply; Hoefer Scientific, San Francisco) for 4 hours with 42

mmol/L of Tris/190 mmol/L of glycine, pH 8.3 at 4°C in the Hoefer Transphor Cell.

In immunostaining with Vectastain ABC kits (Vector Laboratories, Burlingame, CA), nitrocellulose membranes were treated with 1% normal horse serum in PBS, with L10 ascites diluted 1:1,000 in PBS with 3 wt/vol% nonfat dry milk solids (Carnation Co, Los Angeles) (PBS-milk) for 1 hour, washed in PBS-milk, treated with biotinylated horse anti-mouse IgG in PBS-milk, washed with PBS, treated with biotinylated horseradish peroxidase H premixed with avidin DH in PBS-milk, washed with PBS and incubated with 0.002% 4-chloro-1-naphthol and 0.003% H_2O_2 in PBS for 10 minutes.

For L2 immunostaining, the membrane was treated with 1% normal goat serum, with L2 ascites diluted 1:500 in PBS-milk, washed, treated with 0.8 $\mu\text{g}/\text{mL}$ of affinity-purified rabbit anti-mouse IgE antibody (provided by Dr David H. Katz, Medical Biology Institute, La Jolla, CA) in PBS-milk, washed, and treated with biotinylated goat anti-rabbit IgG and detection reagents.

RESULTS

L10 staining of T lineage cells. Various isolated cells of T lineage were analyzed for sialophorin expression by immunoblotting with the anti-sialophorin monoclonal antibody L10. Total peripheral blood lymphocytes, isolated T lymphocytes and thymocytes expressed sialophorin (Fig 1). Total lymphocytes and T lymphocytes display similar amounts of L10-positive material of apparent mol wt 115,000 and minor amounts of diffuse material of slightly lesser mobility. Thymocytes display several-fold less L10-positive material on a "per cell" basis. The L10-positive material in thymocytes is a

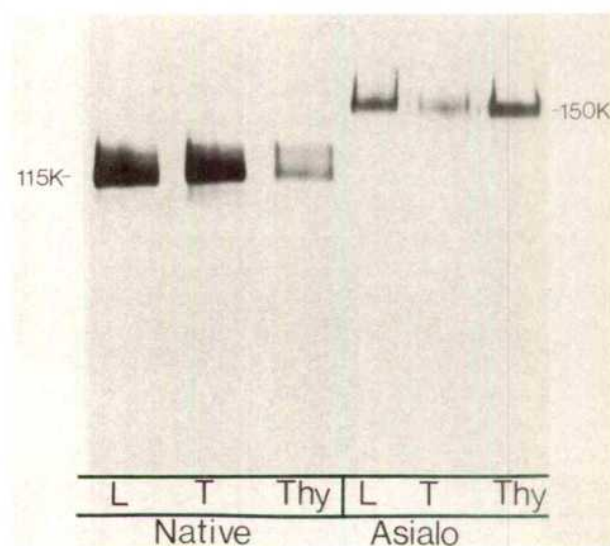


Fig 1. Immunoblot of human T lineage cells stained with L10 antibody. Native and sialidase-treated cell extracts were electrophoresed on Laemmli polyacrylamide gels, transferred electrophoretically to a nitrocellulose membrane, and treated with L10 antibody and immunoperoxidase detection reagents. The L10-reactive native molecules are shown for: L, 10^6 peripheral blood lymphocytes; T, 10^6 isolated T lymphocytes; and Thy, 4×10^6 thymocytes and twice these cell levels for asialo molecules. Arrow on left indicates native sialophorin of lymphocytes (apparent mol wt 115,000); arrow on right indicates asialo-sialophorin (apparent mol wt 150,000).

double band, consisting of the 115,000 component and a regularly detected component of apparent mol wt ~130,000 (Fig 1, lane 3).

Sialidase treatment of T lineage cells. Sialidase treatment converts all L10-reactive species in T lineage cells to the previously described^{2,5} asialo form of apparent mol wt 150,000 (Fig 1), indicating that the heterogeneity of this molecule in T lineage cells is at the level of sialylation. Thus, the minor diffuse material in T lymphocytes and the ~130,000 mol wt component in thymocytes are incompletely sialylated variants of the 115,000-mol-wt molecule.

JY cells, an Epstein Barr virus (EBV)-transformed B cell line, expresses a 115,000-mol-wt L10-reactive molecule (not shown). Untransformed peripheral blood B lymphocytes were not available in sufficient purity and quantity for immunoblotting.

L10 staining of blood cells. Two important observations were made when extracts of various isolated cells were immunoblotted with L10 antibody. First, L10-positive molecules were found not only on lymphocytes, but also on monocytes, neutrophils, and platelets, but not on erythrocytes, fibroblasts, and glioblastoma cells (Fig 2, left panel). Second, two L10-positive molecules were found on normal blood cells. The 115,000 molecule is expressed by lymphocytes and monocytes, and a molecule of apparent mol wt 135,000 is found on neutrophils and platelets (Fig 2, left panel).

Sialidase treatment of blood cells. Sialidase treatment converted the L10-reactive lymphocyte and monocyte molecules to apparent mol wt 150,000 as anticipated (Fig 2, right panel). Sialidase treatment converted the L10-reactive molecule on neutrophils and platelets to apparent mol wt 170,000 (Fig 2, right panel). Thus, variation in sialylation does not account for the difference between the lymphocyte/monocyte and the neutrophil/platelet forms. We further conclude that the lymphocyte/monocyte form and the neutrophil/platelet form have similar content of sialic acid and sialic acid-bearing units because desialylation causes the same unusual quantitative shift in electrophoretic mobility.

Monocytes. In 10 of 15 experiments, monocytes expressed small amounts of the L10-reactive 135,000 mole-

cule in addition to the major 115,000 molecule (not shown). The additional component is not derived from contaminating platelets or neutrophils, as judged by cell staining and by the absence of reactivity of extracts immunoblotted with neutrophil-specific and platelet-specific antibodies (not shown). Monocytes allowed to mature by culturing for 7 days expressed only the 115,000 molecule (13 experiments; not shown).

L2 staining of blood cells. L2 antibody reacts with an epitope on native but not on asialo-sialophorin (Materials and Methods). On immunoblotting with L2 antibody, T lymphocytes and mature monocytes showed a reactive molecule at 115,000; neutrophils and platelets showed a reactive molecule at 135,000 (not shown). Erythrocytes and fibroblasts were negative. The identical staining patterns obtained on native cells with L2 and L10 indicate that the lymphocyte/monocyte molecule and the neutrophil/platelet molecule contain both epitopes.

L10 staining of lymphoblastoid cell lines. L10-reactive molecules were found in native and sialidase-treated T lymphoblastoid cell lines CEM, JURKAT, HPB-ALL, HPB-MLT, HUT-78, and MOLT-4 (Fig 3). Only CEM express the T lymphocyte molecule (native 115,000, asialo 150,000). HUT-78 and MOLT-4 express an L10-reactive molecule like the neutrophil/platelet molecule (native 135,000, asialo 170,000). CEM cells and MOLT-4 were also examined with L2 antibody, and reactive molecules were detected of mol wt 115,000 and 135,000, respectively (not shown). HPB-ALL and HPB-MLT express L10-positive molecules of 135,000 native, 160,000 asialo. The L10-positive molecule of JURKAT with native mol wt 125,000 and asialo mol wt 140,000 differs most from blood cell sialophorin.

Surface location of sialophorin. Whereas sialidase treatment of cell extracts converts all molecules to the asialo form, sialidase treatment of intact cells affects only surface molecules. By the latter treatment, sialophorin of thymocytes, lymphocytes, monocytes, neutrophils, and the lymphoblastoid cells was completely converted to the asialo form (not shown). Thus, sialophorin is exclusively a surface molecule in these cells.

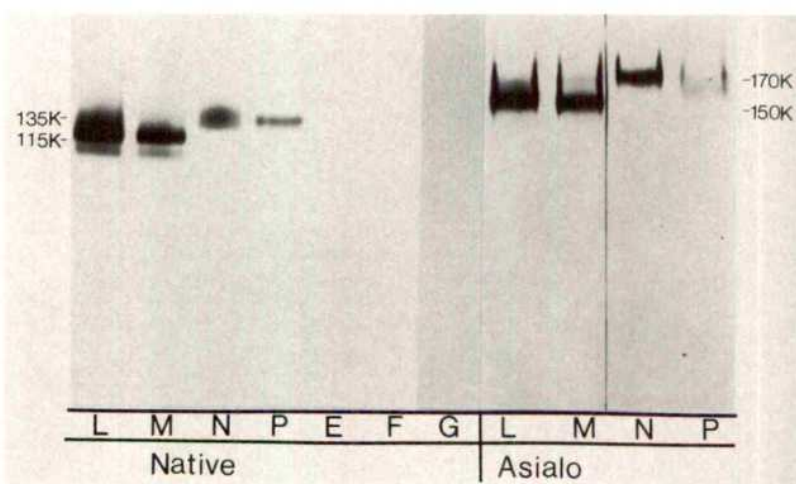
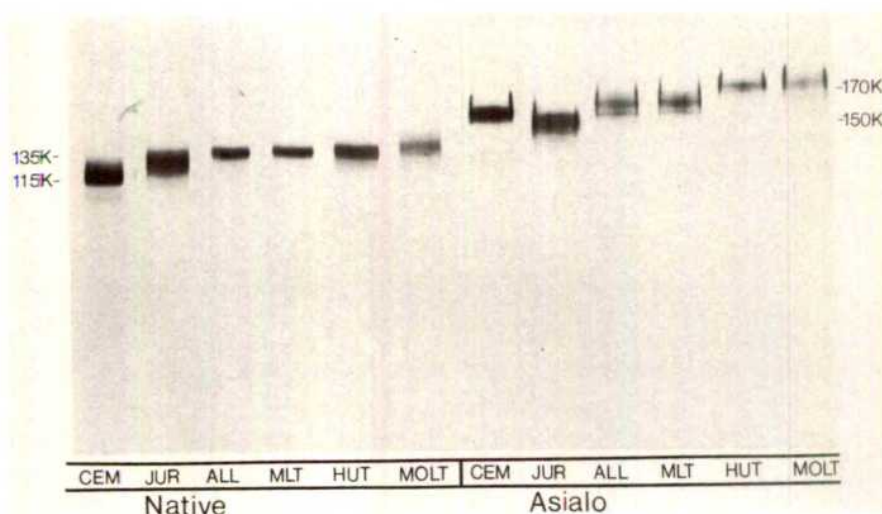


Fig 2. Immunoblot of isolated blood cells and other cells stained with L10. Shown are native extracts of: L, 10^6 lymphocytes; M, 10^6 monocytes; N, 10^6 neutrophils; P, 5×10^7 platelets; E, 50 μ g of erythrocyte "ghost" protein; F, extract of 10^6 MRC-5 normal fetal lung fibroblasts; and G, 10^6 glioblastoma cells (A172). Asialo molecules are for twice these levels of cell extracts. The positions and mol wt (115,000; 135,000) of native sialophorin molecules (left); asialo-sialophorin molecules (right). No L10-positive molecules were detected in native (shown) or asialo (not shown) extracts of erythrocytes, fibroblasts, or glioblastoma cells. Trace components of mol wt <115,000 in native lymphocytes and monocytes result from proteolysis (established in control experiments).

Fig 3. Immunoblot of human lymphoblastoid cell lines stained with L10 antibody. Shown are native (0.5×10^6 cells) and asialo (10^6 cells) extracts of: A, CEM; B, JURKAT; C, HPB-ALL; D, HPB-MLT; E, HUT-78; and F, MOLT-4 cells. Positions of native L10-reactive molecules of CEM cells (115,000 mol wt) and HUT-78/MOLT-4 cells (135,000 mol wt) (arrows, left). Asialo molecules of CEM cells (150,000 mol wt) and HUT-78/MOLT-4 cells (135,000 mol wt) (arrows, right). Only CEM cells express the lymphocyte form of sialophorin.



DISCUSSION

The expression of sialophorin (gpL115) was studied in blood cells. Sialophorin is the sialic acid-rich surface protein, which is altered in the inherited immunodeficiency WAS. Sialophorin was detected on T lymphocytes, thymocytes, B cell lines, monocytes, neutrophils, and platelets, but not on erythrocytes, fibroblasts, and glioblastoma cells. Thus, sialophorin expression appears to be a property of circulating nonerythroid cells and their precursors. This unusual distribution pattern is consistent with the hypothesized function of sialophorin to maintain cells in the circulation (discussed below).

Although their immunoblotting patterns differ, we concluded that thymocytes and T lymphocytes express the same 115,000-mol-wt sialophorin molecule. Small amounts of L10-positive material of lesser mobility in T lymphocytes and a second component in thymocytes were 115,000-mol-wt sialophorin which is incompletely sialylated.

The finding of heterogeneity of sialylation of human thymocyte sialophorin parallels findings with the analogous molecule in the rat called W3/13 antigen or LS GP (leukocyte sialoglycoprotein). LS GP is also less sialylated on thymocytes than on T lymphocytes.¹⁵ The heterogeneity of sialylation of sialophorin and LS GP in thymocytes may reflect the heterogeneity of the cell population.

Two species of L10-reactive molecules were detected on blood cells; these are the previously described mol wt 115,000 species on lymphocytes, thymocytes, and monocytes and the mol-wt 135,000 species on neutrophils and platelets. The 115,000 and 135,000 mol wt species were shown to be substantially similar molecules as follows. Both contain the epitope recognized by L10 antibody and the epitope recognized by L2. Both have approximately the same content of sialylated *O*-linked carbohydrate units since both undergo the same atypical shift in electrophoretic mobility on desialylation.

In a recent study, the polypeptide cores of 115,000-mol-wt sialophorin and 135,000-mol-wt sialophorin were isolated.⁶ The polypeptide cores (mol wt 58,000) were found to be indistinguishable based on chromatographic procedures

including peptide mapping,⁶ thus indicating that the polypeptide portion of the two sialophorin species are identical. We conclude that the mol wt 115,000 and 135,000 species are probably identical except for the presence in the carbohydrate units of the higher mol wt species of additional neutral saccharide residues.

To emphasize the substantial identity of the mol wt 115,000 lymphocyte/monocyte species and the mol wt 135,000 platelet/neutrophil species, we propose that a common name be used. For this reason, we dropped the name gpL115 and replaced it with the name **sialophorin** (sialic acid-bearing molecule). We suggest that the name sialophorin be used for the closely related glycoproteins with different apparent mol wt that are found on lymphocytes, platelets, monocytes, and neutrophils and on precursor hemopoietic cells and related cell lines. Reactivity with L10 monoclonal antibody could be used as a criterion to define sialophorin.

The relatedness of the name sialophorin to the name glycophorin¹⁶ is appropriate because the molecules are chemically related. Both have high content of serine, threonine, and proline, and multiple *O*-linked sialylated carbohydrate units (15 units on glycophorin¹⁷ and ~100 on sialophorin^{5,6}; compositions compared in ref. 5). Glycophorin and sialophorin are reciprocally distributed on circulating blood cells, ie, glycophorin on erythrocytes and sialophorin on platelets, lymphocytes, monocytes, and neutrophils.

The possible relatedness of sialophorin to human lymphoid glycoproteins examined by other investigators was recently summarized (ref. 2, eight studies). Since then, a study has described gp-110, a peanut lectin binding protein of human thymocytes¹⁸ that may be identical to sialophorin. In another study, GP105, the major *O*-glycosidic membrane glycoprotein on EBV-transformed B lymphoblastoid cell lines, which is aberrantly glycosylated in individuals with the Tn blood group, reacted with L10 antibody¹⁹ and therefore meets the criteria for classification as sialophorin. Similarly, gp105, which was also called leukosialin, has multiple properties in common with sialophorin.^{20,21} It is the major sialoglycoprotein on lymphoid and myeloid cell lines and displays varying apparent mol wt. Leukosialin/gp105 molecules should be

classified as sialophorin because they were demonstrated to react with L10 antibody.²⁰ Sialophorin and leukosialin/gp105 are synthesized by *O*-glycosylation of similar size precursors (mol wt 62,000, and 54,000, respectively).^{6,20} Their carbohydrate contents differ, but this is likely due to the choice of cells used as source of the molecules (see below). Their amino acid compositions differ significantly for unexplainable reasons in serine, threonine, proline, and glycine.^{2,20}

Whereas only two forms of sialophorin were detected on circulating blood cells, transformed cell lines express a wide variety of forms of sialophorin (Fig 3 and ref. 20, 21). For example, all T lymphoblastoid cells with the exception of CEM cells express sialophorin species that are different from the 115,000-mol-wt molecule found on circulating lymphocytes (Fig 3). Compositional and lectin analysis of the 115,000-mol-wt sialophorin indicated that the major carbohydrate structures are sialylated units of the disaccharide Gal β 1-3 GalNAc.^{2,5} Direct analysis indicated that the major carbohydrate structures on leukosialin isolated from the "erythroid" cell line K562 are also sialylated Gal β 1-3-GalNAc units.²¹ It thus appears that K562, a cell line from a patient with chronic myelogenous leukemia,²² expresses the sialophorin species found on lymphocytes/monocytes/CEM cells. On the other hand, the oligosaccharides isolated from leukosialin from HSB cells and HL-60 cells (referred to as "myeloid/T-lymphoid leukosialin") are primarily sialylated units of the tetrasaccharide Gal β 1-4 GlcNAc β 1-6(Gal β 1-3)GalNAc,²¹ a structure compatible with the characteristics of platelet/neutrophil sialophorin. Indeed, direct carbohydrate analysis showed that sialylated tetrasaccharides are also the major units on leukosialin of circulating neutro-

phils.²³ It thus appears that "erythroid leukosialin" is the same as lymphocyte/monocyte sialophorin and "myeloid/T-lymphoid leukosialin" as platelet/neutrophil sialophorin.

We hypothesized that sialophorin functions to maintain lymphocytes in the circulation and regulate their orderly senescence and that the central defect in WAS is acceleration of the normal senescence process.^{2,24} This hypothesis was based on consideration of the functional defects in WAS, namely diminished lymphocyte and platelet function,^{3,4} and on the ultrastructural observation of defective surface architecture in WAS lymphocytes.²⁴ We further hypothesize that intact sialophorin functions as a "masking" protein by shielding underlying antigenic sites on other molecules. The structure of sialophorin is particularly suited for this function since it is an extended rod-like molecule bearing ≥ 100 negatively charged sialylated carbohydrate units.^{2,5,6} The loss of sialophorin or the loss of its rod-like structure occurring on removal of sialic acid residues² might expose underlying antigenic sites to which normal circulating antibodies then bind.²⁵ The identification of sialophorin on platelets, neutrophils, and monocytes as well as lymphocytes introduces the possibility that sialophorin functions to maintain various blood cells in the circulation.

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Arginyl-Glycyl-Aspartic Acid Sequences and Fibrinogen Binding to Platelets

By Edward F. Plow, Michael D. Pierschbacher, Erkki Ruoslahti, Gerard Marguerie, and Mark H. Ginsberg

Human fibrinogen has an Arg-Gly-Asp-Ser (RGDS) sequence at residues 572-575 of its A α -chain. Although RGDS-containing peptides inhibit fibrinogen binding to stimulated platelets, these peptides also inhibit platelet binding of human fibrinogen fragment X and rat fibrinogen, which lack RGDS sequences corresponding to A α 572-575. Thus competition between free RGD-containing peptides and internal RGDS sequence at A α 572-575 is not the basis for their inhibition of fibrinogen binding to platelets. Addition of a Thr to the carboxy-terminus and an Asn to the

amino-terminus of the RGDS sequence, the amino acids corresponding to A α 576 and 571 respectively, reduced the inhibitory potency of RGDS-containing peptides by fourfold to tenfold. Arg-Gly-Asp-Phe (RGDF) corresponds to A α 95-98, and the RGDF peptide was an effective inhibitor of fibrinogen binding, fourfold to fivefold more potent than RGDS. Thus, local primary structure may play an important role in regulating the capacity of RGD sequences in proteins to interact with specific adhesion receptors.

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RECEPTOR-MEDIATED recognition of arginyl-glycyl-aspartic acid (RGD) sequences in proteins may constitute a basic and evolutionarily conserved mechanism of cell adhesion.¹ This recognition specificity plays a fundamental role in regulating platelet adhesive reactions. Fibrinogen (Fg), fibronectin (Fn), and von Willebrand factor (vWF) contain at least one RGD sequence. Agonists such as thrombin induce receptors for Fg, Fn, and vWF on platelets, and the interactions of these adhesive proteins with their platelet receptors mediate or modulate platelet attachment, spreading, and aggregation reactions.² RGD-containing peptides inhibit the interaction of all three proteins with these receptors on thrombin-stimulated platelets³⁻⁵ with a concomitant inhibition of platelet adhesive functions.³⁻⁶ Platelet membrane glycoprotein GPIIb-IIIa, which serves as a receptor for Fg, Fn, and vWF, specifically binds to RGD affinity resins.^{7,8} Taken together these data strongly implicate the RGD recognition specificity in mediating the interaction of the adhesive proteins with platelets and in regulating platelet function.

Fg contains two RGD sequences at residues 95-97 and 572-574 of the 610 amino acid A α chain.⁹ The RGD sequence at residues 572-574 is followed by a serine residue at position 575, giving the same tetrapeptide sequence as in Fn¹⁰ and vWF.¹¹ As RGDS-containing peptides partially or fully inhibit^{4,5} the binding of all three adhesive proteins to stimulated platelets, attention has been focused upon the RGD sequence in the carboxy-terminal aspect of the A α chain. In fact, the inhibitory activity of RGDS peptide on Fg binding to platelets has been interpreted to implicate this sequence directly in the interaction.⁵ Such an interpretation

is consistent with the capacity of isolated A α chains to aggregate platelets;¹² the marked decrease in the capacity of plasmic degradation products of Fg, which lack the carboxy-terminal region of the A α chain, to support platelet aggregation;¹³ and the photoaffinity crosslinking of derivatized Fg with intact A α chains to GPIIb-IIIa.¹⁴ Nevertheless it has not been shown that the inhibition of Fg binding by RGD-containing peptide arises from a competition of these peptides with the internal sequence at A α 572-575. In this study we have attempted to directly address this issue.

The RGD sequence A α 95-97 is flanked by phenylalanine at position 98, creating an RGDF sequence.⁹ There is some latitude in the permissive residues at the X position of RGD peptides for adhesive function. For example, peptides with Ser or Val residues have similar activities in inhibiting Fg binding to platelets⁴ and fibroblast attachment to Fn.¹⁰ Indeed the amino acid residue at this position has been found to influence the recognition specificity of different RGD receptors markedly (Pierschbacher MD, Ruoslahti E, manuscript in preparation). Thus as a second issue we have evaluated whether RGDF-containing peptides are active in inhibiting Fg binding to platelets.

MATERIALS AND METHODS

Proteins. Human Fg was purified from fresh plasma by differential ethanol fractionation¹⁵ followed by ammonium sulfate precipitation. Fg-X was produced by digesting Fg with plasmin at a 1:600 (wt/wt) ratio for 30 minutes at 37°C.¹⁶ The plasmin was inhibited with diisopropylfluorophosphate, and the Fg-X was purified by high-performance liquid chromatography (HPLC), as described by Stickland et al,¹⁷ but on a TSK-4000 column. Fg-X migrated as a single broad band on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) under nonreducing conditions with an estimated mol wt of ~250,000. Further characteristics of this fragment are presented in "Results." Fn was isolated from plasma by affinity chromatography on gelatin-Sepharose (Pharmacia Fine Chem, Piscataway, NJ),¹⁸ and vWF was isolated by cryoprecipitation followed by molecular exclusion chromatography.¹⁹ Rat Fg was purified from rat plasma (Pelfreeze Biologicals, Rogers, AR), as described for human Fg. The isolated proteins were radioiodinated by a modified chloramine T procedure, and free ¹²⁵I was removed by dialysis. As previously reported,^{20,22} specific activities range from 0.5 to 2 μ Ci/ μ g, and the precipitability of all radioactive ligands in 15% trichloroacetic acid exceeded 92%.

Peptides. Peptides were synthesized on an Applied Biosystems model 430 peptide synthesizer (Foster City, CA) using PAM resins and t-Boc amino acid purchased from Applied Biosystems. Peptides were cleaved from the PAM resin with HF. The homogeneity of the peptides exceeded 85%, as assessed by reverse-phase chromatogra-

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phy on C18 columns on the HPLC. Peptide concentrations were determined by amino acid analyses of 24-hour hydrolysates in 6N HCl.

Platelet-binding assays. Platelets were isolated from fresh human blood drawn into acid-citrate-dextrose by differential centrifugation followed by gel filtration in modified Tyrode's buffer, pH 7.3, containing 2% bovine serum albumin.²⁰ The binding of the various ligands to the platelets were analyzed as previously described.²⁰⁻²² Briefly, platelets at a final concentration of 1×10^8 /mL were incubated with the radiolabeled adhesive proteins, at their estimated K_d , and varying concentrations of the selected inhibitory reagent. The binding reaction was initiated by addition of the platelet agonist, 10 μ mol/L ADP or 0.5 units thrombin/mL, and the interaction was quantitated after a 30-minute incubation at 22°C for Fg and vWF and 37°C for Fn by separating bound from free ligand by centrifugation of 50 μ L aliquots of the reaction mixture through 20% sucrose. The ID_{50} of a peptide, the concentration of a peptide that produced 50% inhibition of the binding of the radiolabeled ligand observed in the absence of peptide, was determined from dose titration curves. As nonspecific binding, measured by addition of a 50-fold excess of nonlabeled protein, never exceeded 12% of total binding of the ^{125} I-ligands, corrections for nonspecific binding were not employed in these calculations. The ID_{50} of the tetrapeptide RGDS on Fg binding was determined on nine separate occasions with two synthetic products during the course of these studies, and the variation in its ID_{50} value was less than twofold.

SDS-PAGE. Vertical slab gels, 10 cm in length, or cylindrical gels, 0.15×7 cm, were run in the buffer system of Weber and Osborn.²³ Sample preparation and subsequent autoradiography and mol estimations were performed as previously described.²⁴

RESULTS

Effect of RGDS peptides on Fg-X binding to platelets. Fg-X is derived by cleavage of Fg at one or more plasmin-sensitive bonds in the A α 195-239 region as well as at B β 42-43.^{25,26} Thus this fragment contains the amino-terminal aspects of the A α chain but lacks the RGDS sequence at A α 572-575. Fg-X binds to platelets albeit with a lower affinity than Fg.²⁷ The capacity of the RGDS peptide to inhibit the binding of Fg and Fg-X to ADP-stimulated platelets was compared. As shown in Fig 1, RGDS inhibited the binding of Fg and Fg-X to ADP-stimulated platelets. This inhibition was dose-dependent and exceeded 85% for both ligands at peptide concentrations ≥ 200 μ mol/L. The inhibitory potency of the tetrapeptide was similar although not identical for Fg-X and Fg; the ID_{50} values on Fg-X and Fg binding were 40 and 16 μ mol/L respectively. This 2.5-fold difference is reduced to 2.0-fold if the residual interaction of Fg-X at high RGDS concentration is considered to represent nonspecific binding.

To exclude that the observed binding of Fg-X (and inhibitory activity of the peptides on this interaction) was due to selective binding of a minor, residual component of intact Fg in the Fg-X preparation, the cell-bound ^{125} I-radioactivity was analyzed by SDS-PAGE. As shown in Fig 2, the added and bound ^{125} I-Fg-X were identical to one another. The estimated mol wt of the major constituent chains of Fg-X at 48, 41, and 24 kDa are clearly distinguishable from those of the constituent chains of intact Fg at 65, 56, and 48 kDa and are consistent with the known subunit composition of Fg-X.²⁸

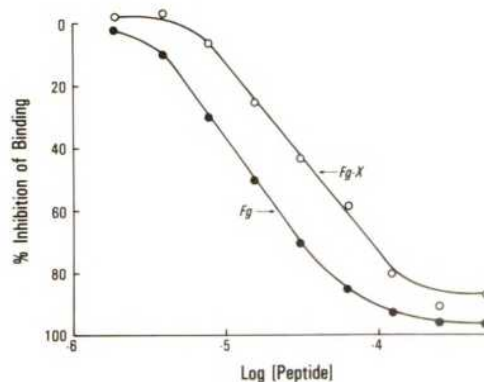


Fig 1. Effect of Arg-Gly-Asp-Ser on binding of fibrinogen fragment X and fibrinogen to ADP-stimulated platelets. ^{125} I-Fg (0.3 μ mol/L) and ^{125} I-Fg-X (1 μ mol/L) were added with varying concentrations of RGDS to washed platelets at 1×10^8 mL in Tyrode's albumin buffer containing 1 mmol/L CaCl_2 . The platelets were stimulated with 10 μ mol/L ADP, and binding was measured after a 30-minute incubation at 22°C. In the absence of the peptide, $14,000 \pm 600$ Fg molecules and $6,000 \pm 200$ Fg-X molecules were bound per platelet.

Effect of RGDS peptides on rat Fg binding to platelets. As an independent approach to examine the competition between the free RGDS-containing peptide and the RGDS sequence in the carboxy-terminus region of the A α chain of Fg, the capacity of RGD-containing peptides to inhibit the binding of rat Fg to human platelets was examined. As determined from cDNA cloning, the protein sequence of the A α chain of rat and human Fg is predicted to be 64% homologous, but the A α chain of rat Fg is shorter:



Fig 2. Characterization of the radioiodinated fragment X added and bound to ADP-stimulated platelets. The ^{125} I-Fg-X preparation was bound to ADP-stimulated platelets for 30 minutes at 22°C. The cell pellets were extracted with 5% SDS, reduced with 2% 2-mercaptoethanol and electrophoresed on a 4% to 10% polyacrylamide gradient gel in SDS under reducing conditions. An autoradiogram of the dried gel is shown. Lane 1 is the added ^{125} I-Fg-X; lane 2 is the ^{125} I-Fg-X extracted from platelets; and lane 3 shows the constituent A α , B β , and γ chains of intact fibrinogen.

531 v 625 amino acid residues.²⁹ This size difference is apparent from the comparison of the two Fg preparations by SDS-PAGE under reducing conditions (Fig 3), and the gels also attest to the purity of the Fg preparations used in this study. According to the alignment of Crabtree et al,²⁹ rat Fg has a deletion corresponding to A α 566-579 and therefore does not contain the carboxy-terminal RGDS sequence. When rat ¹²⁵I-Fg was bound to human platelets stimulated with ADP, RGDS-containing peptides were capable of inhibiting this interaction. As shown in Fig 4, the two RGDS-containing peptides, GRGDSP and RGDS, inhibited the binding of rat Fg to platelets in a dose-dependent fashion. The ID₅₀ values of RGDS on rat and human Fg were 13 and 18 μ mol/L respectively; the ID₅₀ of GRGDSP was 17 and 29 μ mol/L respectively.

Effect of amino acids adjacent to RGDS on adhesive protein binding to platelets. In considering mechanisms that might regulate the interaction of an RGD sequence, such as in the carboxy-terminus of the A α chain, with an RGD receptor, the influence of the amino acids adjacent to the RGDS sequence on peptide inhibitory function was investigated. The sequence of A α 569-577 of human Fg is Ser-Tyr-Asn-Arg-Gly-Asp-Ser-Thr.⁹ As shown in Table 1, attachment of the Thr to the carboxy-terminus of the RGDS sequence resulted in a sixfold higher ID₅₀. The addition of the Asn residue to the amino terminus caused a further diminution in inhibitory potency. Further extension of the peptide in the amino terminal direction by addition of Tyr and Ser-Tyr was without additional effect. Thus the amino acids immediately adjacent to the RGD sequence, particularly at the carboxy-terminus, can have a significant effect in regulating the inhibitory activity of RGD-containing peptides.

RGD sequences are also found within Fn and vWF, and RGD-containing peptides inhibit the binding of these other



Fig 3. Characterization of isolated rat and human fibrinogen by polyacrylamide gel electrophoresis. Lanes 1 and 2 are human (H) and rat (R) fibrinogen respectively, run on cylindrical 5% polyacrylamide gels under nonreducing conditions. Lanes 3 and 4 are human and rat fibrinogen respectively, run on 7.5% polyacrylamide gels under reducing conditions.

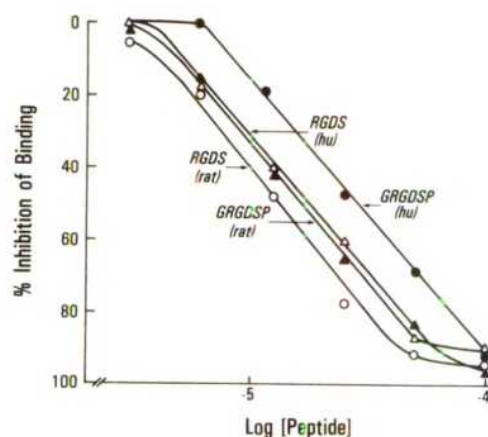


Fig 4. The effect of two RGD-containing peptides on the binding of human (hu) and rat fibrinogen to human platelets stimulated with ADP. Human or rat ¹²⁵I-Fg at 0.3 μ mol/L and varying concentrations of RGDS and GRGDSP were added to platelets at 1×10^8 /mL in Tyrode's albumin buffer containing 1 mmol/L CaCl₂. Platelets were stimulated with 10 μ mol/L ADP, and binding was measured after a 30-minute incubation at 22°C. In the absence of peptides, $18,000 \pm 2,000$ molecules of human fibrinogen and $8,000 \pm 800$ molecules of rat Fg bound per platelet.

two adhesive proteins to platelets.³⁻⁵ In view of the marked effects of the amino- and carboxy-terminal residues adjacent to the RGDS sequences on Fg binding, the influence of the adjacent residues in the sequences of Fn and vWF was evaluated. As the analysis of Fn binding requires the use of thrombin-stimulated platelets, the effect of the peptides tested above on Fg binding to adenosine diphosphate (ADP)-stimulated platelets were also tested on its binding to thrombin-stimulated platelets. As indicated by a comparison of Tables 1 and 2, the potency of the individual peptides Arg-Gly-Asp-Ser (RGDS), Arg-Gly-Asp-Ser-Thr (RDGST), and Asn-Arg-Gly-Asp-Ser-Thr (NRGDST) as inhibitors of Fg binding to either ADP or thrombin-stimulated platelets was similar. For example, the ID₅₀ values for RGDS on Fg binding to ADP- and thrombin-stimulated platelets were 22 and 24 μ mol/L respectively. The sequence of the cell attachment site in Fn is Gly-Arg-Gly-Asp-Ser-Pro.¹⁰ Addition of a Pro to the carboxy-terminus or a Pro at the carboxy-terminus and a Gly at the amino-terminus did not affect the ID₅₀ values of these peptides on Fn binding. These results are consistent with

Table 1. Effect of RGDS and the Amino Acids Adjacent to A α 572-575 on Fibrinogen Binding to ADP-Stimulated Platelets

Peptide	ID ₅₀ (μ mol/L)
RGDS	22
RGDST	125
NRGDST	200
YNRGDST	220
SYNRGDST	200

¹²⁵I-Fg at 0.3 μ mol/L and varying concentrations of the indicated peptides were incubated with platelets, 1×10^8 mL and 10 μ mol/L ADP for 30 minutes at 22°C in Tyrode's buffer containing 1 mmol/L CaCl₂. In the absence of peptides $16,500 \pm 200$ Fg molecules were bound per platelet. The ID₅₀ values are the means from triplicate determinations in two experiments performed with different platelet donors.

Table 2. Effect of the Amino Acids Adjacent to the RGDS Sequences in the Adhesive Proteins on Their Binding to Thrombin-Stimulated Platelets

Adhesive Protein	Peptide	ID ₅₀ (μmol/L)
Fibrinogen	RGDS	24
	RGDST	82
	NRGDST	230
Fibronectin	RGDS	15
	RGDSP	14
	RGDST	18
	GRGDSP	8
vWF	RGDS	18
	PRGDSQ	29

¹²⁵I-Fg (0.3 μmol/L); ¹²⁵I-Fn (0.3 μmol/L) or ¹²⁵I-vWF (400 ng/mL) were incubated with 1×10^8 platelets/mL, varying concentrations of the indicated peptides, and 0.1 units/mL thrombin for 30 minutes in Tyrode's albumin buffer containing 1 mmol/L CaCl₂. In the absence of peptides, the molecules/platelets of Fg, Fn, and vWF were $17,500 \pm 3000$, $29,600 \pm 4000$ molecules/platelet; and 700 ± 50 ng/10⁹ platelets, respectively.

previous data reported from our laboratories.³ Similar results were found with vWF, where the sequence of the RGD region is Pro-Arg-Gly-Asp-Ser-Gln. Attachment of a Gln to the carboxy-terminus and a Pro to the amino-terminus only had a slight effect on the potency of the peptides as inhibitors of vWF binding to platelets. Thus in contrast to Fg, extension of the RGDS sequence in the amino- and carboxy-terminal direction had minimal effect on the capacity of the RGDS-containing peptides to inhibit Fn and vWF binding to platelets. Also included in Table 2 is the effect of RGDST on Fn binding to platelets. Although this peptide was considerably less potent than RGDS in inhibiting Fg binding, it had a potency similar to RGDS on Fn binding. This suggests that the variable inhibitory activity of the peptides is, at least in part, established by the particular adhesive protein interacting with the cells.

Effect of RGDF peptides on Fg binding to platelets. The second RGD sequence in the Aα chain is contained within the peptide Leu-Arg-Gly-Asp-Phe-Ser (LRGDFS), which corresponds to Aα 94-99. We have also used synthetic peptides to evaluate the effect of a Phe residue adjacent to the Asp residues and of the additional carboxy- and amino-terminal amino acids on the activity of this RGD region. As shown in Table 3, RGDF is an inhibitor of Fg binding to ADP-stimulated platelets and is in fact approximately fourfold to fivefold more potent than RGDS. Attachment of a Ser residue to the carboxy-terminus to generate RGDFS caused a ninefold decrease in inhibitory activity based on the ID₅₀ values. Extension of the peptide in the amino-terminal direction with the naturally occurring residue Leu or with a Gly had little additional effect on inhibitory activity. In comparing the inhibitory activities of the hexapeptides corresponding to Aα 94-99 and Aα 571-576, the former peptide is fourfold more potent. The increased potency of the RGDF relative to RGDS was also observed with Fg binding to thrombin-stimulated platelets and with Fn and vWF as ligands. The ID₅₀ values of RGDF on Fn and vWF binding were 2 and 3 μmol/L, respectively.

Table 3. Effect of the RGDF Sequence and Adjacent Amino Acids on Fibrinogen Binding to ADP-Stimulated Platelets

Peptide	ID ₅₀ (μmol/L)
RGDF	4
RGDFS	36
LRGDFS	44
GRGDFS	48

Experimental details are the same as in Table 1. In the absence of peptides, $18,200 \pm 600$ Fg molecules were bound per platelet.

DISCUSSION

Synthetic peptides with structures corresponding to small segments of larger proteins have been highly useful in dissecting the recognition specificities of a variety of cell-surface receptors^{10,30,31} including the receptors for platelet adhesive proteins.^{3-6,32} As originally established for Fn³ and more recently demonstrated for Fg and vWF,^{4,6} RGDS-containing peptides inhibit the binding of these adhesive proteins to their inducible platelet receptors. As each of these adhesive proteins contains an RGDS sequence, direct competition between the free RGDS peptides and the RGDS sequences in the proteins is a likely mechanism for the observed inhibition. The data shown in the present study indicate the situation may be more complex in the case of Fg. Namely, the interaction of two Fg forms, human Fg fragment X and rat Fg, which both lack an RGDS sequence corresponding to Aα 572-576 of human Fg, was inhibited by RGDS-containing peptides. Moreover the potency of the RGDS-containing peptides in inhibiting the binding of these Fg forms lacking the RGDS sequence was similar to that observed with intact human Fg containing the RGDS sequence. These observations are consistent with the results of Peerschke and Galanakis,³³ who found that intact fibrinogen and derivatives lacking the carboxyterminus of the Aα chain exhibited similar binding affinities for platelets. It should be emphasized that these results do not exclude that the RGDS sequence at Aα 572-576 of human Fg interacts directly with the platelet. However, competition between the sequence in the protein and the free peptide cannot be the sole basis for the inhibitory activity of the free peptides.

As alternatives to the direct competition between free and protein-contained RGDS sequences, four possibilities may be considered. First, the free peptide may bind to Fg (or the adhesive proteins) and prevent its binding to platelets. This possibility seems unlikely, as the platelet receptor for the adhesive proteins, GPIIb-IIIa, binds directly to RGDS coupled to a solid support,⁷ whereas Fg and Fn do not. Second, RGDS-containing peptides may bind to the platelet and may alter the capacity of the receptor to interact with the adhesive proteins. Such an allosteric mechanism of inhibition cannot be excluded. However, RGD peptides inhibit Fg binding to platelets on which the receptor has been fixed in an induced state with paraformaldehyde,⁴ and the peptides appear to affect receptor affinity rather than the number of binding sites for the adhesive proteins.³ Third, the basis for the inhibitory capacity of the RGDS peptides may rest in their competition with the RGDF sequence at Aα 95-97. This may explain why certain Fg derivatives that contain an

RGDF sequence such as the N-DSK and certain fragment E preparations can inhibit (albeit weakly) the binding of Fg to platelets.^{34,35} Fourth, the RGDS peptides may compete with the carboxy-terminus of the γ chain of Fg for the platelet recognition site(s). Peptides corresponding in structure to the carboxy-terminus of the γ chain of Fg (having the structure LGGAKQAGDV) also inhibit Fg as well as Fn and vWF binding to platelets.^{21,32} We have recently found that both γ chain and RGD-containing peptides coupled to Sepharose bind GPIIb-IIIa and can elute the glycoprotein bound to an affinity column of the other peptide.³⁶ Thus by competing for the same site(s) the RGD peptides could inhibit Fg binding to GPIIb-IIIa mediated by the γ chain sequence, and the RGD sequences in Fg need not interact directly with platelets.

To consider potential mechanisms that might regulate the function of the RGDS sequence in Fg, we examined the effect of the amino acids adjacent to the RGDS sequence on the inhibitory potency of peptides as antagonists of Fg binding to platelets. Indeed the adjacent amino acids significantly influenced the potency of the peptides. RGDST was a considerably less potent inhibitor of Fg binding than RGDS, and NRGDST was still less potent. Thus the local sequence surrounding the RGD residues may play an important role in regulating the interaction of RGD sequences with RGD receptors. This effect of local sequence may preclude an interaction of Fg with platelets via A α 572-576 and may also be the basis for the apparent lack of interaction of Fg with certain cells expressing RGD receptors.^{10,37} In contrast to such very local effects, the overall conformation of Fg might determine the availability of the A α 572-576 sequence. However, the carboxy-terminal of the A α chain is predicted to be highly hydrophilic and flexible.³⁸ We have also subjected A α 551-611 to the secondary structure predictive analyses (Chou-Fasman Program) available at the University of Wisconsin Genetics Computer Group Program.³⁹ The RGDS sequence at A α 572-576 is predicted to be hydrophilic and involved in β turns, according to the predictive analyses of Hopp and Woods⁴⁰ and Chou and Fasman⁴¹ respectively. Similar predictions are also derived for the RGDS sequence within Fn. Thus these predictive analyses provide no basis to

postulate a unique conformation to the carboxy-terminal aspects of the A α chain of Fg which would interfere with the accessibility of this RGDS sequence. Therefore very local effects such as those established by the amino acids immediately adjacent to the RGDS sequence could play an important regulatory role. The amino acids adjacent to the RGDS sequences in Fn and vWF had a minimal effect on the inhibitory potency of RGD-containing peptides on the binding of these other two adhesive proteins to platelets. These data are compatible with a direct role of these RGDS sequences in mediating Fn and vWF binding to platelets. It is also noteworthy that, as emphasized by comparison of the inhibitory potency of RGDST on Fg and Fn binding to thrombin-stimulated platelets (ID₅₀ differences of approximately fivefold), that the same peptide can have differential effects on the binding of the various adhesive proteins to platelets. This provides evidence that the platelet receptors for these adhesive proteins may not be precisely identical. The differences in the ID₅₀ values of the RGD-containing peptides on Fg v Fg-X in Fig 1 and rat and human Fg in Fig 4 is most readily explained in terms of the possibility of more than one recognition site within these ligands, which results in nonidentical binding of the adhesive protein to the platelet.

Finally, it was noted that RGDF was a particularly potent inhibitor of Fg as well as Fn and vWF binding to platelets. This raises the possibility that peptide or nonpeptide analogues based on the RGD sequence can be constructed that could be very potent inhibitors of platelet function. Initial evidence for this has been provided by the publication of Ruggeri et al.⁴² The potential of such analogues as anti-thrombotic agents would depend upon their selectivity for platelets.

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Second Marrow Transplants in Patients With Aplastic Anemia Rejecting the First Graft: Use of a Conditioning Regimen Including Cyclophosphamide and Antithymocyte Globulin

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Sixteen (11%) of 146 consecutive patients with severe aplastic anemia prepared for engraftment with cyclophosphamide (200 mg/kg) rejected marrow grafts from their HLA-identical siblings. They were given a second marrow transplant from either the same ($n = 13$) or a second ($n = 3$) HLA-identical sibling between 23 and 743 (median 86) days after the first transplant. The preparation for the second transplant included cyclophosphamide, 50 mg/kg, on each of four successive days. Twelve hours after each of the first three doses of cyclophosphamide, antithymocyte globulin, 30 mg/kg/dose, was infused. One of the 16 patients died from infection too early after the second transplant to be evaluated, two had failure of engraftment and died with infection, one rejected the second graft and is surviving almost 5 years later with full autologous marrow recovery, and 12 had successful and sustained second grafts. Of these 12, six are surviving between 11

months and 7 $\frac{3}{4}$ years. Four of the six have no graft-v-host disease (GVHD), while two have chronic GVHD requiring treatment. Five have Karnofsky scores of 100% and one of 90%. Six of the 12 patients with sustained grafts died between 63 days and 38 months after transplantation, four with infections (related in two patients to chronic GVHD), one with acute GVHD, and one with hemorrhage. The average interval from first to second transplant was 308 days during the past five years, compared to 61 days in earlier patients. Five of seven recent patients are surviving, compared to two of nine earlier patients. In conclusion, successful second transplants after cyclophosphamide and antithymocyte globulin are possible in most patients with aplastic anemia who have rejected their first marrow grafts; however, mortality remains high, with only 40% of the patients becoming long-term survivors.

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GRAFT REJECTION is associated with a high mortality in patients with severe aplastic anemia given marrow grafts from HLA-identical family members.¹⁻⁶ We reported earlier that only two of 29 patients undergoing graft rejection became long-term survivors.⁷⁻⁹ One of the surviving patients showed full restoration of autologous marrow function, and one had a successful second transplant. Isolated additional cases of successful second transplants have been described.¹⁰⁻¹³ Various conditioning regimens for second transplants have been used, including cyclophosphamide (CY) and total body irradiation (TBI), or procarbazine and antithymocyte globulin (ATG) combined with CY or TBI.⁷⁻¹⁵ All of these regimens had associated toxicities, and in many cases the second graft was not established. Based on a murine study showing synergism between CY and ATG,¹⁶ we first explored alternating CY and ATG to condition a patient who had rejected two preceding marrow transplants.⁷ The success seen in this patient led us to adopt CY/ATG as our conditioning regimen for patients who have rejected their first

graft. We now report its use in 16 consecutive patients with aplastic anemia.

MATERIALS AND METHODS

From October 25, 1977 to August 23, 1985, 146 patients with severe aplastic anemia were transplanted with marrow from their HLA-identical family members following conditioning with CY. Details of the first transplants in these patients have been reported previously.¹⁷⁻¹⁹ Sixteen of the 146 patients (11%) either failed to show evidence of initial marrow engraftment or rejected the first graft. Characteristics of these 16 patients are shown in Table 1. Marrow donors and recipients were HLA-A and HLA-B identical siblings whose cells were mutually nonreactive in mixed leukocyte culture.

Patients were referred after detailed consultation with their physicians and treated after outpatient and inpatient conferences that fully outlined the advantages and disadvantages of the transplantation procedure. Protocols and consent forms were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center.

Before first transplantation all 16 current patients received CY, 50 mg/kg intravenously (IV) on each of four successive days. Thirty-six hours after the last dose of CY, donor marrow was infused IV. The day of first marrow infusion was designated day 0. Five of the 12 multiple-transfused patients were also given viable donor buffy-coat cells as reported previously.¹⁸ The four untransfused patients and seven of the 12 multiple-transfused patients did not receive buffy-coat cell infusions. These infusions are not routinely given in untransfused patients¹⁷ nor in transfused patients under the age of 18 years²⁰; six of the transfused patients were in this young age group, while one was transplanted before buffy-coat cell transfusions became part of the transplantation protocol.

Postgrafting immunosuppression consisted of methotrexate in nine, cyclosporine in one, and a combination of methotrexate and cyclosporine in six cases, as previously described.¹⁹

The conditioning for second transplant consisted of CY, 50 mg/kg IV on each of four successive days. At 12-hour intervals following the first, second, and third dose of CY, patients also were given ATG (ATGAM, Upjohn Company, Kalamazoo, MI), 30 mg/kg IV infused over a period of 10 to 12 hours. Marrow for second

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Table 1. Patient Data at Time of First Marrow Transplant

Characteristic	Datum
No. of patients studied	16
Age (yr), median (range)	14 (5-37)
Sex (F/M), (no. of patients)	6/10
Possible causes of aplastic anemia (no. of patients)	
Unknown	13
Drug or chemical	2
Pregnancy	1
Duration of aplastic anemia in months, median (range)	1.8 (0.2-14.9)
No. of untransfused patients*	4
Preceding transfusions in units, † median (range)	
RBCs	15 (1-multiple)
Platelets	86 (4-multiple)
Refractory to random donor platelets (no. of patients)	3
History of treatment (no. of patients) with:	
Antithymocyte globulin	1
Androgens	6
Prednisone	8
Positive relative response index in MLC‡ (no. of patients)	2

*These patients did not receive transfusions earlier than 72 hours before CY.

†In many cases exact information on the transfusion history could not be obtained; the patients' records merely indicate that multiple transfusions had been administered.

‡Response of patient cells to sibling cells $\geq 2.6\%$ of that to cells from unrelated individuals.⁴⁶

transplant was infused 36 hours after the last dose of CY. In 13 cases marrow infusion for the second transplant was supplemented by donor buffy-coat cell infusions. In three cases marrow donors were too young to serve as buffy-coat cell donors.

Assessment of hematopoietic engraftment and grading and treatment of acute and chronic GVHD were done as previously described.²¹⁻²³ Primary therapy of established grades 2 to 4 acute GVHD consisted of methylprednisolone, 2 mg/kg/day, either IV or orally in divided doses for a period of seven to 14 days when the patient's status was re-evaluated. Chronic GVHD was treated with methylprednisolone, either alone or combined with azathioprine (Aza). Marrow aspirates and/or cytogenetic studies usually were performed on days 14, 21, 28, 56, 84, and 365 postgrafting to assess the quality of engraftment.

Results of the study were analyzed as of August 1986.

RESULTS

Tables 2 and 3 summarize the transplantation data and results.

First transplant. Two of the 16 patients failed initial marrow engraftment, while 14 showed rises in granulocyte counts and evidence of marrow cellularity on day 14. Granulocyte counts rose to above 1,000 at a median of 20 days, not different than previously reported for patients with sustained marrow engraftment. In eight of the 16 patients the marrow donor was of opposite sex. Cytogenetic studies in these eight showed predominance of donor type cells in the marrow on day 14 and subsequent occasions, while phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells showed a mixture of host and donor karyotypes. An example of cytogenetic findings for one of the patients is shown in

Table 2. Transplantation Data

Characteristic	First Marrow Graft	Second Marrow Graft
Donor age (yr), median (range)	14 (1-41)	16 (2-40)*
Sex, F/M (no. of individuals)	5/11	7/9*
Median (range) of marrow cells infused ($\times 10^{-8}$ /kg)	2.9 (1.0-6.7)	2.8 (1.5-12.3)
No. of patients given additional buffy-coat infusions from marrow donor	5	13
Median (range) number of buffy-coat cells infused ($\times 10^{-8}$ /kg)	3.3 (1.0-12.3)	6.5 (0.7-20.5)
No. of patients in laminar airflow room	13	16
Postgrafting immunosuppression (no. of patients)		
Methotrexate	9	9
Cyclosporine	1	1
Methotrexate/cyclosporine	6	6
Median (range) days to granulocytes $> 1,000/\mu\text{L}$	20 (19-35)	27 (15-158)

*In three instances a different HLA-identical sibling was used as marrow donor for the second graft.

Table 4. Rejection was characterized by progressively falling peripheral blood counts, decreasing marrow cellularity, and the appearance of increasing numbers of lymphocytes of host origin demonstrated by cytogenetic analysis after PHA stimulation. The decision to proceed with a second transplant was prompted by the development of severe marrow aplasia.

Second transplant. Second grafts were carried out between 23 and 743 (median 86) days after the first grafts. In all but three cases the same HLA-identical sibling was used as the marrow donor for the second transplant. The median numbers of marrow cells infused in the first and second grafts were comparable. The postgrafting immunosuppression in the second was the same as that used in the first transplant. Acute toxicity associated with ATG administration included skin rash (four patients), fever (eight patients), and hypotension (two patients). One patient developed signs of serum sickness consisting of rash, fever, and arthralgias beginning eight days after ATG infusion.

Following the second graft, one patient died too early to be evaluated (day 4 after second graft), two patients failed to engraft and died with infection (days 18 and 39), and one patient showed initial engraftment followed by graft rejection. The latter patient showed complete recovery of his own marrow and is alive more than 57 months after the first marrow graft with normal hemopoietic function. Twelve patients showed sustained engraftment. Of these 12, six developed acute GVHD grades 2 to 4, two of whom died from mixed bacterial/viral and fungal pneumonia (days 91 and 193) and four of whom developed chronic GVHD. Two of the four with chronic GVHD died with bacterial infections (days 987 and 1085), and two are surviving at 85 and 93 months, one with a Karnofsky score of 100% and one of 90%, both requiring immunosuppressive therapy. Five patients did

Table 3. Results of Second Marrow Grafts

Characteristic	Datum
Median (range) days between first and second grafts	86 (23-743)
Fate of graft (no. of patients at risk = 16)	
Not evaluable	1
Failure of engraftment	2
Rejection (and full autologous recovery)	1
Sustained engraftment	12
Acute GVHD (no. of patients at risk = 12)	
Grade 0	5
Grade 1	1
Grade 2	2
Grade 3	3
Grade 4	1
Chronic GVHD (no. of patients at risk = 10)	
Yes	4
No	6
Causes of death (n = 9)	
Graft failure and/or bacterial infection	5
Acute GVHD	1
Chronic GVHD and related infections	2
Hemorrhage	1
Surviving patients (n = 7)	
Entirely well (one with autologous marrow recovery)	5
Chronic GVHD (one with myasthenia gravis also)	2
Median (range) months of follow-up from first transplant	49 (11-93)

Table 4. Cytogenetic Studies in Female Patient, UPN 906, First Given A Marrow Graft From a Sister With Down's Syndrome and Then a Graft From a Healthy Brother

Day After Grafting	No. of Metaphases Studied					
	Marrow (Not PHA Stimulated)			Peripheral Blood (PHA Stimulated)		
	XX	XX + G	XY	XX	XX + G	XY
0 first BMT	10			20		
14	5	7				
26				7	8	
29	2	15				
33				14	15	
40				13	12	
43	1	22				
48				2	3	
55				15	10	
57	2	9				
62				7		
69	49	1				
71				9		
76				53		
83				18		
90	20					
103 second BMT						
12						20
14			34			
18-1460			75			109

This patient has chronic GVHD and also myasthenia gravis.⁴⁷

Abbreviations: PHA, phytohemagglutinin; BMT, bone marrow transplant.

not develop acute GVHD, and one had grade 1 acute GVHD. One of these six died with bladder hemorrhage related to thrombocytopenia on day 63, one with pseudomonas aeruginosa pneumonia on day 393, and four are living between 11 and 50 months after the first transplant. Cytogenetic and other blood genetic marker studies in surviving patients showed donor type hematopoiesis in six cases and complete host-type hemopoiesis in one.

The three young patients not given supplemental buffy-coat cell infusions all engrafted and are surviving without evidence of acute or chronic GVHD. All three patients given a second marrow graft from a different donor engrafted, and one is surviving at 7½ years with chronic GVHD.

Over the past five years rejections occurred generally at a later time than was seen before the middle of 1981; this was true both for methotrexate- and cyclosporine-treated patients. Accordingly the interval between first and second transplant increased on the average from 61 to 308 days. Five of seven patients in the latter group are surviving, compared to two of nine in the former group.

Figure 1 illustrates the cumulative probability of survival for these 16 patients as measured from the day of first transplant.

DISCUSSION

By the mid 1970s, graft rejection was recognized as a frequent complication after marrow transplantation for severe aplastic anemia, occurring in 35% to 70% of patients given HLA-identical marrow grafts following conditioning with CY.^{1-3,6,24} Rejection is likely to be caused by sensitization of recipient cells to minor histocompatibility antigens expressed on donor cells through preceding blood transfusions. During the past decade the incidence of rejection has declined to 17% in the series reported by the European and International Bone Marrow Transplant Registries^{4,5} and to 11% in patients transplanted in Seattle.^{18,25,26} The reduction in the frequency of rejection has been attributed both to more aggressive treatment regimens, including TBI, total lymphoid or thoracoabdominal irradiation, cyclosporine,^{4,5,27-32} and the addition of buffy coat cells to the marrow inoculum,¹⁸ and to changes in the quantity and quality of preceding blood transfusion products.^{17,26}

Except in those few patients who have subsequent recovery of autologous marrow function,³³⁻³⁸ graft rejection has remained a serious complication owing to the return of marrow aplasia. For second transplants to be successful, graft rejection needs to be diagnosed promptly and distinguished from other causes of marrow malfunction. Reversible marrow hypoplasia in the setting of a marrow graft can be caused by certain viral infections and also by drugs such as trimethoprim sulfamethoxazole, acyclovir, interferon, or methotrexate.³⁹ Blood genetic markers are most helpful in distinguishing between rejection and marrow suppression. Mixed chimerism (mixtures of cells with host and donor karyotype) has been seen in a majority of patients (58%) early after marrow grafting, while a minority of patients (42%) never showed host cells after transplantation.⁴⁰ Rejection was seen almost exclusively in patients with mixed

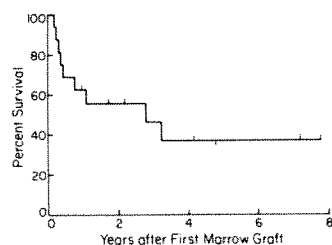


Fig 1. Probability of survival (measured from first marrow graft) in 16 patients with severe aplastic anemia given second marrow transplants from HLA-identical siblings (Kaplan-Meier product limit estimates).⁴⁶ Tic marks above the line represent surviving patients with sustained engraftment; the tic mark below the line represents the patient with autologous marrow recovery. Survival is as of August 1986.

chimerism (30% incidence) and was rare in complete chimeras (5% incidence). Persistence of mixed chimerism and/or gradual disappearance of donor type cells, in particular among PHA-stimulated peripheral blood lymphocytes, may be the first signs of graft rejection. Molecular biology techniques, such as restriction-enzyme fragment-length polymorphism studies, will be useful in those patients whose donors are sex matched and who lack distinctive banding markers.⁴¹

Once a diagnosis of graft rejection has been established in the presence of marrow aplasia, conditioning for second transplantation should proceed. We have previously reported second marrow transplants using a variety of regimens in 21 patients carried out 26 to 157 days after the first transplant.^{7,9} Six of these 21 attempts were not evaluable because of early deaths, eight were unsuccessful, and seven were successful with only one of the 21 patients now surviving for more than 11½ years. Eight additional patients with rejection were not given a second transplant; seven of these died with infection and marrow hypoplasia, and one is alive with complete autologous marrow recovery 12 years later.³³ Thus of 29 patients undergoing rejection, only two are surviving.

Isolated successful transplants have been described by other transplant centers.¹⁰⁻¹³ Previous conditioning regimens included combinations of CY and TBI, procarbazine/ATG combined with TBI or CY, and, in one instance, the present combination of ATG and CY. The current experience using this combination proved to be more successful than our past experience with other conditioning regimens, and 12 of 15 evaluable patients showed sustained engraftment. Presumably, the addition of ATG to CY usually provides sufficient immunosuppression to overcome sensitization to minor histocompatibility antigens expressed on donor cells that lead to rejection of the first graft, although a contribution of added buffy-coat cell infusions cannot be excluded. The regimen was generally well tolerated, apart from fevers and chills associated with the ATG infusion. While successful in securing sustained engraftment, the regimen did not prove to be uniformly life saving. Half of the patients with engraftment developed significant acute and/or chronic GVHD and died from associated infectious complications. However, six patients are living with sustained grafts, five with Karnofsky scores of 100% and one of 90%, although two of the six have

chronic GVHD requiring treatment. Graft rejection is presumed to be due to histocompatibility differences involving "minor" antigens. These same differences may account for GVHD when a persisting marrow graft is established, as was observed in half of the cases described here, which may contribute to the mortality.

Over the past five years rejections occurred generally at a later time (on the average ten months after grafting) than were seen before the middle of 1981 (on the average two months after grafting). Results with second transplants in the recent patients were better than those in the earlier group. The reasons for the recent increase in the time to rejection are unclear. Hows et al⁴² described late graft failure in 13% of their cyclosporine-treated patients following discontinuation of immunosuppressive therapy and suggested that cyclosporine had delayed what otherwise might have been early rejection.²⁹ This explanation may be true for some of our patients; however, others have received methotrexate during the first three months after marrow transplantation. An alternative explanation for the late graft failure seen in some of the present patients is the re-emergence of a population of abnormal, immunologically reactive cells (presumably T cells) that were responsible for creating the original aplastic anemia. Evidence that such cells may exist in patients with aplastic anemia is based on previous *in vitro* observations, the response of some patients to treatment with ATG, and the occasional failure of syngeneic marrow grafts to take in the absence of conditioning with CY.^{43,44} In the present patients the increased immunosuppression provided by the combination of ATG and CY may have abolished the abnormal T cell population, thereby setting the stage for sustained allogeneic marrow engraftment. In the absence of *in vitro* assays distinguishing between immunity to an allograft caused by sensitization through blood products and "autoimmunity," it remains unclear which of these mechanisms was operative in the late rejection seen in the present patients.

One of the three patients with failure of sustained engraftment showed complete recovery of autologous marrow function, a finding described previously in isolated patients.³³⁻³⁸ Whether the ATG contained in the second conditioning regimen contributed to this recovery remains conjectural.

In conclusion, while graft rejection has become a lesser problem than it was a decade ago, it remains a significant complication after marrow transplantation for patients with severe aplastic anemia. Early recognition of graft rejection through careful hematologic follow-up and the use of blood genetic markers may permit successful second transplantation after conditioning with ATG and CY, resulting in sustained and stable hematopoietic engraftment.

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Loss of Myeloid Differentiation Antigens Precedes Blastic Transformation in Chronic Myelogenous Leukemia

By Mary B. Todd, James A. Waldron, Timothy A. Jennings, Lisa S. Rome, Sanford D. Markowitz, Theodore R. Holford, Joseph P. Gardner, John P. Wolak, and Harry L. Malech

In order to determine whether antigenic patterns alter with disease progression and are thereby suggestive of impending blast crisis in chronic myelogenous leukemia, 50 bone marrow biopsy specimens from 32 patients were examined retrospectively using indirect immunoperoxidase labeling with three monoclonal antibodies that detect myeloid antigens. Monoclonal antibodies PMN13F6, PMN7C3, and PMN8C7 detect human neutrophil antigens that first appear at the myeloblast, promyelocyte, and metamyelocyte stages of differentiation, respectively, and persist throughout later differentiation. Percentages of antigen-positive bone marrow cells during the chronic phase were compared with percentages of antigen-positive cells at blast transformation, and time from bone marrow biopsy until blast crisis was correlated with the percentage of bone marrow cells expressing these antigens. Bone marrow biopsy samples from patients in the chronic phase who continue to remain clinically stable 4 to 106 months after biopsy expressed PMN13F6 antigen on $82\% \pm 9\%$ (mean $\pm$ SD) of cells, PMN7C3 antigen on $62\% \pm 14\%$ of cells, and PMN8C7 on $68\% \pm 14\%$ of cells. Bone marrow biopsy specimens obtained from patients 1 or more years prior to blast transformation expressed PMN13F6 antigen on $81\% \pm 12\%$, PMN7C3 antigen on $71\% \pm 16\%$, and PMN8C7 on $64\% \pm 16\%$ of cells. Bone marrow biopsy samples obtained between 2 months and 1 year prior to blast crisis expressed PMN13F6 antigen on $68\% \pm 15\%$, PMN7C3 on $51\% \pm 17\%$, and PMN8C7 antigen

on $46\% \pm 18\%$ of cells. Bone marrow biopsy specimens taken at the time of blast transformation expressed PMN13F6 antigen on $20\% \pm 25\%$, PMN7C3 antigen on $19\% \pm 25\%$, and PMN8C7 antigen on $13\% \pm 25\%$ of cells. The difference between the mean of antigen-positive cells from bone marrow biopsy samples obtained at the time of blast crisis was significant compared with the mean of positive cells from biopsy specimens obtained at all other phases of the disease ($P < .001$ for all three antibodies). There was a positive correlation between loss of myeloid antigens and disease progression as determined by simple regression of log time and correlation analysis (PMN13F6, $r = .6533$, $P < .005$; PMN7C3, $r = .6304$, $P < .005$; PMN8C7, $r = .5215$, $P < .05$). There was a negative correlation between percentage of immature cells and time to blastic crisis ($r = -.6206$, $P < .005$). There was no correlation between the percentage of immature cells present prior to blastic transformation and expression of the antigen appearing at the metamyelocyte stage detected by PMN8C7 ($r = -.3145$, $P < .20$). There was an inverse relationship between the percentage of immature cells and presence of the early appearing antigens detected by PMN7C3 ($r = -.5144$, $P < .05$) and by PMN13F6 ($r = -.7961$, $P < .001$). The emergence of increasing numbers of cells that do not express certain myeloid differentiation antigens in addition to morphological changes may serve as an indicator of impending blast transformation.

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CHRONIC MYELOGENOUS LEUKEMIA (CML) is a myeloproliferative disorder with two distinct phases. The first (chronic) phase usually is controllable until progression to the second phase (accelerated or blastic), which is a more aggressive form that is universally fatal in a short time despite treatment with chemotherapy. The median duration of the chronic phase ranges between 2 and 4 years but is extremely variable, with some patients presenting in

the blast transformation stage and others remaining in the chronic phase for many years.¹⁻³

Successful transplantation of bone marrow to individuals with CML has resulted in long-term survival of individuals with this previously fatal disorder.⁴ However, transplantation after blast transformation or during the accelerated phase has been disappointing due to failure to eradicate the leukemia, incomplete engraftment, or additional complications of bone marrow transplantation.⁴⁻⁶ Best results are obtained when patients receive transplants during the chronic phase. However, because transplantation itself has at least a 30% risk of death,⁴ it is appealing to delay bone marrow transplantation until as late as possible in the course of the disease.

Studies by several groups have helped to define prognostic signs that identify patients at the time of diagnosis of CML who are at high risk for early progression to the second phase.⁷⁻¹¹ Other studies have helped to define signs that may indicate transition from the chronic phase to the blastic phase.¹²⁻¹⁵ Prognostic indicators noted at diagnosis that suggest poor prognosis are well documented (ie, lack of a Philadelphia chromosome)^{9,16,17} but fail to provide an indicator of impending transition for an individual patient. Signs such as fever, splenomegaly, increasing anemia, thrombocytopenia, and basophilia are either not specific for an impending aggressive phase or are noted too late in the course of the disease to be of benefit. The appearance of increasing numbers of myeloblasts and promyelocytes in blood or bone

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marrow is commonly used as a sign of disease progression, but this criterion alone is often not specific for the time course until that individual patient enters blastic crisis.

In this study we correlated changes in the expression of neutrophil differentiation antigens in bone marrow cells with transition to a more aggressive phase of CML. Fifty bone marrow biopsy specimens from 32 patients were examined retrospectively by using indirect immunoperoxidase labeling with three monoclonal antibodies that detect myeloid differentiation antigens. Our findings indicate that there is a significant difference in the percentage of cells that expressed these antigens at the time of blastic crisis compared with other phases of the disease, that antigen loss preceded blastic crisis, and that there was a correlation between the percentage of antigen-positive cells and time until transformation. Antigenic changes, in combination with other indicators and signs of disease progression, may be useful for the prediction of impending blastic crisis.

MATERIALS AND METHODS

Patients. After appropriate human protection committee validation, paraffin-embedded bone marrow biopsy specimens from patients with the diagnosis of CML were obtained from the Yale-New Haven Hospital Pathology Department. Normal bone marrow samples were obtained when biopsies were done for purposes other than this study, and the results were interpreted as normal by conventional light microscopy. All patients had been advised of the procedure of bone marrow biopsy and the alternative risks of that procedure, in accordance with institutional guidelines, and gave informed consent. Patients were chosen on the basis of availability of adequate bone marrow biopsy material and confirmation of the diagnosis of CML. The charts of all patients were reviewed. The diagnostic criteria for CML were based on conventional criteria including history and physical, a typical peripheral blood and bone marrow picture with increased myeloid elements, and when available, chromosomal analysis and leukocyte alkaline phosphatase score. Differential counts of peripheral blood smears were previously performed by technicians of the clinical hematology laboratory of this hospital. Histochemical studies of bone marrow biopsy and aspirate samples were reviewed. All bone marrow biopsy sections were stained with hematoxylin and eosin for purposes of this study and were examined by two pathologists. Bone marrow samples were evaluated for features such as myeloid-erythroid ratio and percentage of myeloblasts, promyelocytes, and myelocytes.

Monoclonal antibodies. Hybridomas that secrete antihuman neutrophil antibodies were obtained by using a variation of the method of Kohler and Milstein¹⁸ as previously noted.^{19,20} In brief, BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized with purified normal human neutrophils,²¹ and spleen cells from these mice were fused with SP2/0 Ag14.²² Hybridomas producing antibodies that bind to human neutrophils were cloned by limiting dilution.

Experiments were conducted with monoclonal antibody obtained from filtered spent media from growing hybridoma cultures or from filtered ascites of BALB/c mice injected with hybridomas. Determination of immunoglobulin subclass was done by Ouchterlony analysis²³ using subclass-specific rabbit antimouse immunoglobulin.

Binding of monoclonal antibodies to blood cells. Human neutrophils were separated from heparinized venous blood from normal donors by using Ficoll-Hypaque (Histopaque 1083, Sigma Chemical Co, St Louis) density gradient centrifugation followed by dextran sedimentation and hypotonic lysis of erythrocytes.²¹ Mononuclear cells were separated by using Ficoll-Hypaque density gradient

centrifugation. For some studies mononuclear cells were further separated on a reorienting Percoll gradient to obtain a fraction containing more than 95% monocytes and no neutrophils.²⁴ Cell purity was assessed by Wright-Geimsa stain.

The binding of monoclonal antibodies to blood cells was measured by using an indirect enzyme-linked immunoassay (Bethesda Research Laboratories, Gaithersburg, MD) as previously described.^{19,20} The binding of monoclonal antibodies was also assessed by direct or indirect immunofluorescence as previously described.²⁵ Cells were directly observed (Zeiss microscope) or analyzed by using a Becton Dickinson (Mountain View, CA) FACS IV to quantify binding.

Biochemical characterization of antigens detected by monoclonal antibodies. Neutrophils isolated from the peripheral blood of normal donors were incubated with 2 mmol/L diisopropyl fluorophosphate (DFP; Aldrich Chemical Co, Milwaukee) for one hour at 4°C to decrease proteolysis.²⁶ Neutrophils were then treated with extraction buffer (18 mmol/L Tris-glycine, pH 8.5, 0.5% Triton X-100, 5 mmol/L EDTA, 20 mg/mL chymostatin [Sigma], 5 mmol/L DFP, 5 mmol/L 2-mercaptoethanol) for ten minutes on ice. After centrifugation, the supernatant extract was used for electrophoretic analysis. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), extracts were mixed with an equal volume of SDS solubilizing buffer (6% SDS, 6 mol/L urea, 125 mmol/L Tris-HCl, pH 6.9, 4 mmol/L EDTA, 5% 2-mercaptoethanol) and electrophoresed following the method of Laemmli²⁷ with some changes in conditions as previously described.²⁰ Molecular weight determinations (M_r) were extrapolated from curves by using protein standards of known molecular weight. Isoelectric focusing (IEF) or nonequilibrium pH gel electrophoresis (NEPHGE) of cell extracts was done as previously described.^{20,28,29} Cell extract was mixed with an equal volume of IEF solubilizing buffer (8 mol/L urea, 2.5% ampholines of pH 3 to 10 [Bio-Rad Laboratories, Richmond, CA], 1.5% Triton X-100, 0.5 mol/L B-mercaptoethanol) and isoelectric focused into polyacrylamide tube gels containing urea, Triton X-100, and ampholines. After IEF or NEPHGE the gels were extruded into SDS solubilizing buffer and incubated for 30 minutes. The gels were then placed onto polyacrylamide slab gels for SDS-PAGE in the second dimension.

Antigens separated by SDS-PAGE or two-dimensional PAGE were electrophoresed onto nitrocellulose paper and detected by immunoblot (Western blot) analysis by sequential use of the murine monoclonal antibodies, rabbit antimouse Ig, and ¹²⁵I staphylococcal protein A as previously described.^{30,31} Nonspecific antibodies of the same subclass or culture supernatants from a non-immunoglobulin-secreting mouse myeloma cell line were used as controls.

Detection of antigen expression in bone marrow biopsy tissue. Tissue sections from formalin-fixed, paraffin-embedded bone marrow biopsy specimens were processed for immunoperoxidase staining by a variation of a published method.³² Sections on albumin-coated slides were extracted with xylene to remove the paraffin, washed in ethanol, and treated with 1% hydrogen peroxide in methanol to inactivate endogenous peroxidase activity. Sections were rehydrated, treated with 0.1% pronase in Tris buffer, washed in Tris-saline, and exposed to 20% normal rabbit serum or 20% normal swine serum for 30 minutes to decrease nonspecific binding of antibodies. Sections were exposed for one hour to 1:50 dilutions of ascites containing specific monoclonal antibodies or control ascites. Polyclonal rabbit antilysozyme (Dakopatts Antibodies, Accurate Chemical and Scientific Corp, Westbury, NY) was used to examine the lysozyme content of the cells. After washing, the sections were exposed either to peroxidase-conjugated rabbit IgG antimouse immunoglobulin or to peroxidase-conjugated swine IgG antirabbit immunoglobulin (Dakopatts Antibodies, Accurate Chemical and Scientific Corp). The peroxidase reaction was developed with 3,3-

diaminobenzidine (Sigma) and the sections counterstained with hematoxylin. Bone marrow biopsy specimens were reviewed by light microscopy, and the percentage of peroxidase-positive bone marrow cells was determined by four observers. The total percentage of cells that expressed a particular antigen was determined regardless of whether cells appeared to be blastic, more mature myeloid, or nonmyeloid in morphology.

Correlation of antigenic expression and blast transformation. The following, when accompanied by appropriate clinical presentation, was accepted as the definition of the blastic stage of CML: 25% or more blasts in peripheral blood or bone marrow, 40% or more blasts plus promyelocytes in blood, or 50% or more blasts plus promyelocytes in marrow. In addition, the presence of leukemic tumor masses or tissue infiltration with immature leukemic cells was accepted as evidence of extramedullary blastic transformation.

The two-tailed Student's *t* test was used to determine the significance of differences in the percentage of antigen-positive cells in marrow biopsy specimens from different subgroups of patients. Linear regression and correlation analysis were used to assess the significance of the relationship between the percentage of cells expressing a particular antigen and the time from the date of biopsy to the date of blast transformation. The form of the relationship between the two variables was explored with a scatter plot. Three patients included in this analysis had more than one bone marrow biopsy sample obtained 2 months or more prior to blast transformation. Multiple biopsy specimens from the same patient may not be statistically independent; however, there is possible bias in eliminating either early or late observations. Therefore, the data were analyzed by both including these multiple biopsy samples or excluding the early or late biopsy samples from these three patients. Linear regression and correlation analysis were also used to assess the relationship between the percentage of immature cells (myeloblasts and promyelocytes) in bone marrow specimens and the time until blastic transformation and the relationship between the percentage of immature cells and the percentage of cells expressing a particular antigen.

RESULTS

Characteristics of the patient population. Thirty-two patients are included in this study. All patients had the diagnosis of CML made by standard criteria including bone marrow. Two patients were diagnosed prior to transfer of their care to Yale-New Haven Hospital, and peripheral blood counts obtained prior to the initiation of single-agent therapy were not available. None of the patients in this study presented in blast crisis, although two patients had greatly increased blasts plus promyelocytes (40% to 50%) in the bone marrow at time of diagnosis. Both patients were treated with hydroxyurea, and both remain in the chronic phase at 1 and 3 years after diagnosis. Cytogenetic studies were done on all but one patient. Three patients were negative for the Philadelphia chromosome, whereas the remainder were all positive. Three patients had additional chromosomal abnormalities detected at the time of diagnosis. The subpopulation of patients that were Philadelphia chromosome-negative or had additional chromosomal abnormalities at the time of diagnosis is too small for individual analysis. There are 17 females and 15 males, with a median age of 52.7 (range, 25 to 89). The mean time elapsed between the onset of symptoms and diagnosis was 2.5 months. All patients were treated with conventional therapy consisting of single-agent chemotherapy (hydroxyurea or busulfan) during the chronic stage.

Clinical and laboratory features of the 32 cases at the time of diagnosis are shown in Table 1.

Of the 32 patients, 20 (63%) have died due to progression of CML into the blastic or accelerated phase, and 12 (37%) were alive at the last reporting date. The actuarial survival curve for this population (Fig 1) is consistent with other patterns previously described for CML patients.^{2,7-9} Eight patients who entered blast transformation developed chromosomal abnormalities in addition to the Philadelphia chromosome during the course of the disease. In six patients, these changes were detected at the time of blast transformation. Additional chromosome abnormalities were noted 14 months and 5 months prior to blast transformation in the remaining two patients. Chromosomal abnormalities in addition to the Philadelphia chromosome have been noted in three patients who remain in the chronic phase at 64 months, 15 months, and 12 months of follow-up.

Characterization of the monoclonal antibodies and the antigens. Monoclonal antibodies from three hybridoma subclones, PMN13F6 (IgG₁), PMN7C3 (IgG₃), and PMN8C7 (IgG₁) were chosen for this study because they detected antigens that first appear at different stages of neutrophil differentiation. PMN7C3 has been described in detail elsewhere.^{19,20}

The results of an enzyme-linked indirect immunoassay used to quantitate the binding of the monoclonal antibodies to peripheral blood cells are shown in Fig 2. All three monoclonal antibodies were neutrophil specific with no significant binding to mixed mononuclear cells (lymphocytes plus monocytes), monocytes, or erythrocytes. None of the antigens identified by these three monoclonal antibodies were expressed on any cells other than neutrophils and

Table 1. Clinical and Laboratory Features at the Time of Original Diagnosis

		Number of Patients	Percentage*
Hemoglobin (g/100 mL)	<8	2	6.6
	8-2	20	66.7
	>12	8	26.7
WBC ($\times 1,000$)	<30	5	16.7
	30-100	9	30.0
	>100	16	53.3
Platelet count ($\times 1,000$)	<150	0	0
	150-500	18	60.0
	>500	12	40.0
Blood myeloblasts	None	12	40.0
	1-5	13	43.3
	>5	5	17.2
Philadelphia chromosome	Positive	28	90.3
	Negative	3	9.7
Spleen	Not palpable	9	29.0
	Palpable	22	71.0
Liver	Not palpable	16	51.6
	Palpable	15	48.4
Symptoms prior to diagnosis	Absent	8	25.0
	Present	24	75.0

*The percentage of patients within each subcategory is based on the total number of patients for which information regarding that category is known.

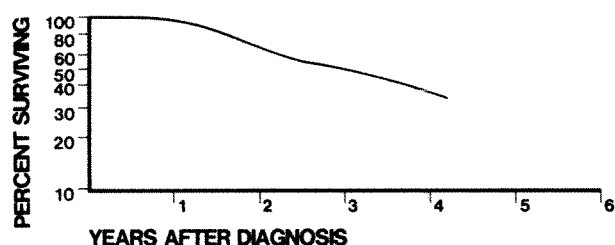


Fig 1. Survival curve of all patients included in study from the date of diagnosis.

neutrophil precursors as determined by indirect immunofluorescence labeling followed by cell cytometry. Direct visualization of indirect immunofluorescence-labeled cells confirmed that none of the three antibodies bound to eosinophils, basophils, monocytes, lymphocytes, platelets, or erythrocytes. In addition, using these same techniques, it was determined that all mature, normal neutrophils expressed the antigens detected by PMN13F6 and PMN8C7. A subset of 1% to 3% of neutrophils from some individuals did not express the antigen identified by PMN7C3.

Normal bone marrow biopsy material exposed to monoclonal antibodies PMN13F6, PMN7C3, and PMN8C7 and subsequently labeled by the indirect immunoperoxidase method is shown in Fig 3. There were no peroxidase-positive cells after incubation with control ascites (Fig 3A). PMN13F6 (Fig 3B) bound to all neutrophil precursors in normal marrow. Myeloblasts and all more mature neutrophils were peroxidase-positive when labeled with PMN13F6, whereas islands of erythroid precursor cells and megakaryocytes remained negative. PMN7C3 (Fig 3C) labeled promyelocytes and all more mature neutrophils. PMN8C7 (Fig 3D) bound to metamyelocytes, band forms, and mature neutrophils.

These antibodies detected antigens present in whole cell extracts from normal neutrophils. The antigens were examined after both single-dimension SDS-PAGE (Figs 4 and 5) to determine the molecular weight and IEF focusing followed by PAGE to determine pI (data not shown). PMN13F6

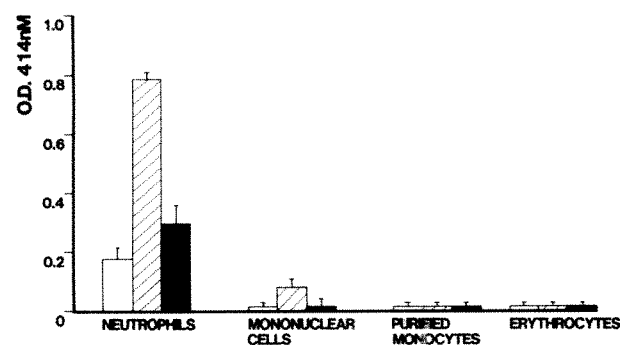


Fig 2. Binding of (□) PMN13F6, (▨) PMN7C3, and (■) PMN8C7 to human peripheral blood cells measured in an indirect enzyme-linked antibody microtiter assay. Binding is expressed as the optical density at 414 nm of the enzyme reaction product. The results shown are the mean with SE of binding to an equal number of neutrophils, mononuclear cells, or erythrocytes from each of three donors.

detected two bands of material with an M_r of 68,000 and 80,000 and a pI of 4.5 to 5.4 when examined by Western blot analysis (Fig 4). PMN7C3 recognized a myeloid-specific carbohydrate epitope present on a number of neutrophil glycoproteins including the group of related leukocyte membrane glycoproteins associated with the C3bi receptor complex.²⁰ PMN7C3 appeared to detect two broad, high-molecular weight bands (Fig 4) on Western blot analysis of whole cells. PMN8C7 detected a membrane antigen that consists of a single band with an M_r of 14,000 to 16,000 and a pI of 7.8 to 8.3 (Fig 5).

Expression of antigens on bone marrow cells from patients with CML. Analysis of antigen expression on bone marrow biopsy specimens from the 20 patients in the study who have progressed to blast crisis is shown in Table 2. Ten of these bone marrow biopsy samples were obtained from the patients at time of the initial diagnosis of CML and prior to single-agent therapy. An example of bone marrow biopsy material obtained from a patient at the time of diagnosis and labeled with PMN13F6 is shown in Fig 6A. The marrow is hypercellular, and the majority of cells expressed the antigen detected by PMN13F6 antibody. There was a similar increase in antigen-positive cells with PMN7C3 and PMN8C7 at the time of diagnosis of CML as compared with antigen-positive cells in normal bone marrows.

The remainder of the biopsy specimens represented in Table 2 were obtained at various stages throughout the chronic phase or blastic phase. An example of a bone marrow biopsy sample obtained at the time of blastic transformation and labeled with PMN13F6 is shown in Fig 6B. The marrow was occupied predominately by cells that do not express the antigen. There was a similar pattern of loss of expression of the antigens identified by PMN7C3 and PMN8C7 in bone marrow biopsy specimens obtained at the time of blast crisis.

In all cases with multiple sequential biopsy samples, except one, there was a decrease in the percentage of cells expressing the antigens detected by these antibodies as the disease progressed to the terminal stage (Table 2). Loss of antigen expression occurred in both mature- and immature-appearing myeloid cells (Fig 6), and loss of antigen expression occurred in both lymphoid (terminal deoxynucleotidyl transferase [TdT]-positive) and myeloid (TdT-negative) blast transformation (Table 2). The one case that did not exhibit a decrease in the percentage of cells expressing these antigens (case number 8) was a patient with extramedullary blast transformation diagnosed from axillary lymph node biopsy material.

The percentage of binding of PMN13F6 to all bone marrow biopsy specimens obtained at the time of blast transformation, excluding the patient with extramedullary blast transformation, ranged from no binding to 40% binding (mean $\pm$ SD, $14\% \pm 12\%$), and including the patient with extramedullary blast transformation, the mean percentage of binding was $20\% \pm 25\%$ (Table 3). Bone marrow biopsy specimens obtained from patients more than 2 months but less than 1 year prior to the time of blast transformation were 50% to 90% positive for PMN13F6 (mean, $68\% \pm 15\%$), whereas those obtained 1 year or more prior to blast transfor-

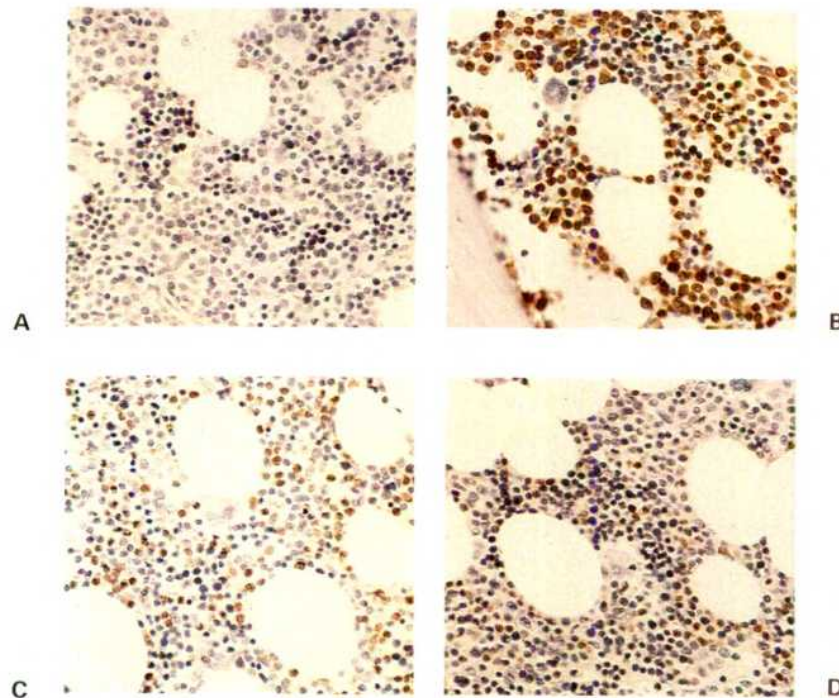


Fig 3. Sections of a normal bone marrow biopsy sample labeled with the immunoperoxidase method using control mouse ascites-derived immunoglobulin (3A), PMN13F6 (3B), PMN7C3 (3C), or PMN8C7 (3D). The peroxidase reaction product is brown; nuclei are stained purple. Cytoplasm is not counterstained to avoid obscuring the peroxidase stain. In Fig 3A (control), there are no peroxidase-positive cells. PMN13F6 incubation, Fig 3B, followed by immunoperoxidase resulted in labeling of the myeloblasts as well as all the more mature neutrophil cells and precursors. PMN7C3, Fig 3C, detects an antigen present on promyelocytes and all more mature neutrophils; PMN8C7 detects an antigen that first appears at the metamyelocyte level.

mation were 50% to 95% positive for PMN13F6 (mean, $81\% \pm 12\%$).

Sixteen bone marrow specimens from 12 patients who have not yet entered the accelerated or blastic phase of disease were examined for antigenic expression of normal myeloid antigens. The median follow-up is 30 months (range, 4 to 106 months), and the mean binding for PMN13F6 is $82\% \pm 9\%$ (Table 3).

The two-tailed Student's *t* test was used to determine the statistical significance of differences in the percentage of antigen-positive cells in biopsy samples obtained at different time intervals. The difference between the mean of PMN13F6-positive cells from bone marrow biopsy material obtained at the time of blast transformation, even including the patient with extramedullary blast transformation in the evaluation, was highly significant compared with the means of all other categories ($P < .001$). The mean of PMN13F6-

positive cells from biopsy material obtained 2 months to 1 year prior to blast transformation was less significant compared with the mean of positive cells from bone marrow specimens taken more than 1 year before blast crisis ($P < .06$) but was significant compared with the mean of PMN13F6-positive cells from patients who have not yet entered blastic transformation ($P < .01$). The difference between the mean of PMN7C3- ($19\% \pm 25\%$) and PMN8C7 ($13\% \pm 25\%$)-positive cells from biopsy specimens at the time of blast transformation was highly significant compared with the means of all other categories ($P < .001$). The mean of PMN7C3- and PMN8C7-positive cells from biopsy material obtained 2 months to 1 year prior to blastic transformation was significant compared with the means of positive cells from biopsy samples taken more than 1 year before blast crisis ($P < .05$).

A scatter diagram expressing the relationship between the

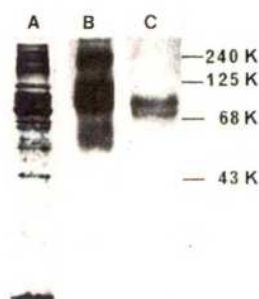


Fig 4. SDS-PAGE (10% acrylamide) of a membrane-enriched fraction of human neutrophils. Figure 4A shows gel stained with Coomassie blue. Electrobots from this gel with immunoblot identification of the antigens detected by PMN7C3 (4B) or PMN13F6 (4C) are shown in adjacent panels.

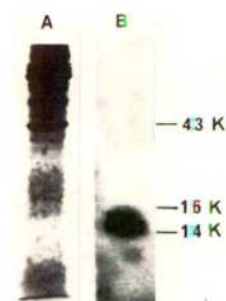


Fig 5. SDS-PAGE (13% acrylamide) of a membrane-enriched fraction of human neutrophils stained with Coomassie blue (5A); electroblot immunoblot identification of antigens detected by PMN8C7 antibody (5B).

Table 2. Percentage of Bone Marrow Cells Binding to Monoclonal Antibodies, Percentage of Myeloid-Appearing Cells, Percentage of Immature Cells, and the Time from Bone Marrow Biopsy to Blast Transformation in Patients Who Are Now Dead

Patient No. (Biopsy No.)	PMN13F6	PMN7C3	PMN8C7	Myeloid Cells	Immature* Cells	Months to Blastic Crisis
1 (1†)	90	70	70	60	<1	45
(2)	10	5	5	90	80	0
2† (1)	90	80	70	80	5	13
(2)	60	70	50	80	40	3
(3)	5	10	10	0	80	0
3 (1)	50	25	50	80	40	3
(2)	40	20	20	90	60	0
4 (1)	75	40	15	90	20	5
(2)	5	5	5	95	90	0
5 (1†)	90	70	50	80	10	11
(2)	5	5	0	90	80	0
(3)	70	50	10	60	20	—
6 (1†)	90	60	60	80	5	14
(2)	5	5	0	90	80	0
(3)	60	10	10	60	40	—
(4)	10	0	0	80	80	—
7 (1†)	75	90	80	80	10	29
(2)	60	70	80	90	45	5
8 (1†)	50	50	50	80	10	6
(2)	95	95	95	90	70	0
9 (1†)	95	90	90	85	<5	49
(2)	90	85	50	80	<5	35
(3)	50	30	30	90	45	14
10 (1)	20	20	10	80	60	0
11 (1)	65	30	30	90	35	6
12 (1†)	80	75	70	70	10	27
13† (1)	0	0	0	10	90	0
14† (1)	10	5	5	10	90	0
15 (1)	20	25	0	80	50	0
16 (1†)	80	70	70	80	25	23
17 (1†)	80	60	60	80	5	22
18§ (1)	30	30	10	70	10	0
19§ (1)	90	50	40	90	<5	9
20 (1†)	70	65	50	90	30	20

*Myeloblasts plus promyelocytes except for those samples obtained at the time of blastic crisis in which all blast cells, regardless of lineage, were included.

†Bone marrow biopsy material obtained at time of diagnosis of CML prior to single-agent therapy.

‡Terminal deoxynucleotidyltransferase-positive blast cells at the time of blast crisis.

§Philadelphia chromosome-negative.

||Bone marrow biopsies done after transformation to blast crisis and after aggressive chemotherapy (not included in statistical analysis).

percentage of bone marrow cells expressing the PMN13F6 antigen and the time to blast transformation is shown in Fig 7. Only bone marrow biopsy specimens obtained more than 2 months prior to blastic transformation are included in this evaluation ($n = 19$) in order to determine the value of antigen expression as a predictor of blastic transformation. Regression and correlation analysis determined that the correlation coefficient was significant ($r = .6533$, $P < .005$) for PMN13F6. As noted in Materials and Methods, three patients had more than one biopsy sample obtained prior to

blast transformation. If the multiple observations from these three patients were excluded from the analysis, as described in Materials and Methods, the correlation coefficient was still significant ($P < .05$). The correlation coefficient for the relationship between the percentage of cells expressing the antigen detected by PMN7C3 from all specimens prior to blastic crisis and the time until blast transformation was $r = .6304$ ($P < .005$), and for the relation of PMN8C7 binding to time of blast transformation it was $r = .5215$ ($P < .05$).

Regression and correlation analysis of bone marrow samples obtained within 1 year of blastic transformation indicated a significant relationship between the percentage of PMN13F6-positive cells and the time to blastic crisis ($r = .7739$, $P < .02$). Because of the small number of bone marrow biopsy specimens ($n = 8$), further analysis of this category was not done.

Regression and correlation analysis of the relationship between antigen-positive cells and time to blastic transformation was also determined for all biopsy material from patients who have progressed ($n = 31$) including those samples obtained at the time of transformation (time 0). The correlation coefficient was highly significant ($P < .001$) for the relationship between expression of all three antibodies and time of blastic transformation (PMN13F6, $r = .8167$; PMN7C3, $r = .7665$; PMN8C7, $r = .7436$).

The relationship of the percentage of cells positive for each of the three antibodies and the percentage of myeloblasts and promyelocytes present in each bone marrow sample obtained prior to blastic transformation was analyzed. There was a negative correlation between the percentage of cells reactive with PMN13F6 and the percentage of immature cells ($r = -.7961$, $P < .001$). Analysis of the relationship between the percentage of cells positive for PMN7C3 and percentage of immature cells resulted in a correlation coefficient of $r = -.5144$ ($P < .05$). There was no significant relationship between PMN8C7 positivity and the number of immature cells ($r = -.3145$, $P < .20$) by correlation and regression analysis.

Regression and correlation analysis of the percentage of immature cells present in bone marrow biopsy material obtained prior to blastic crisis and the time until blastic crisis determined a significant correlation coefficient of $r = -.6206$ ($P < .005$).

Three bone marrow samples from two patients were examined after aggressive combination chemotherapy following blastic transformation. Therapy consisted of daunorubicin, cytosine arabinoside, and 6-thioguanine. Bone marrow biopsy specimen 3 from patient 5 (Table 2) was obtained 6 weeks after beginning aggressive therapy and was interpreted as consistent with a return to the chronic phase. The patient relapsed 4 weeks later and expired within 1 week. Bone marrow biopsy specimen 3 from patient 6 was obtained 1 month after the beginning of aggressive therapy and was interpreted as a 50% decrease in blasts from the examination done at the time of blastic crisis. A subsequent bone marrow biopsy sample (no. 4) demonstrated repeated antigenic loss 4 weeks later when the disease progressed despite therapy. This patient failed to respond to additional aggressive chemotherapy and died 2 weeks after the last biopsy.

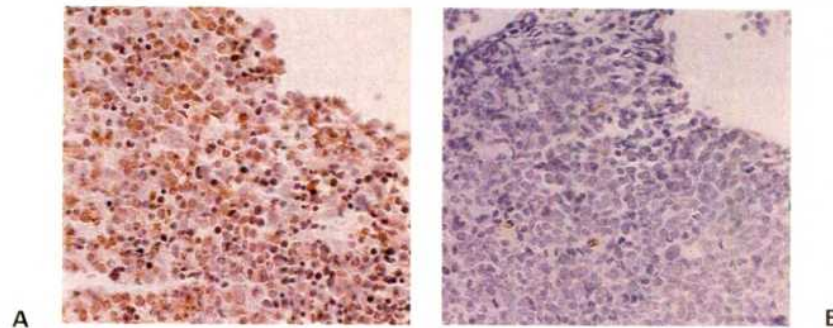


Fig 6. Bone marrow biopsy specimens obtained from patients at the time of diagnosis of CML (6A) and blastic crisis (6B). Both were labeled by using the immunoperoxidase method using PMN13F6, the earliest-appearing myeloid antigen used in this study. In Fig 6A (diagnosis) the marrow is hypercellular, and the majority of cells express the antigen detected by PMN13F6. In Fig 6B (blastic crisis), the marrow shows predominantly immature cells that, although myeloid, do not express the PMN13F6 antigen. In addition, some mature myeloid cells failed to express the PMN13F6 antigen.

Intracytoplasmic lysozyme was examined in all bone marrow biopsy material by using the antilysozyme antibody. Myeloid cells at various stages of differentiation, from promyelocytes to segmented forms, were strongly positive for intracytoplasmic lysozyme. Blast cells from patients at all stages showed great variability in amount of lysozyme detected.

DISCUSSION

Studies by several groups have examined the prognostic significance of disease features recorded at the time of diagnosis.⁷⁻¹¹ These include the presence or absence of the Philadelphia chromosome, spleen size, percentage of circulating blasts, major abnormalities of erythropoiesis or platelet production, and basophilia or eosinophilia. The Philadelphia chromosome has prognostic significance as an indicator of survival but is not useful for predicting the timing of blast transformation for an individual patient.^{9,14,16,17} A high proportion of myeloblasts plus promyelocytes in peripheral

blood or bone marrow at diagnosis have been shown to indicate a shortened overall survival time.⁷⁻¹⁰ However, again these criteria are not reliable predictors of the time from diagnosis to blast transformation for an individual patient.

A number of signs acquired during the clinical course of CML are suggestive of impending transformation to the blastic stage.¹²⁻¹⁵ The appearance of blastic crisis is often associated with chromosomal aberrations in addition to the Philadelphia anomaly.¹⁴ Additional chromosomal abnormalities were found in cells from some subjects included in our study. In the majority of cases, these were not detected until obvious blastic transformation. Chromosomal abnormalities often occur too late in the course of the disease to be of value for the prediction of the time to blastic crisis. The appearance of two Philadelphia chromosomes during the course of the disease signals impending blastic crisis,¹⁷ but this event is too rare to be predictive in the majority of patients.^{14,17}

Table 3. Percentage (Mean $\pm$ SD) of Bone Marrow Cells Expressing Myeloid Antigens at Different Intervals Prior to Blast Crisis, at Blast Crisis, or From Patients Who Remain in the Chronic Phase

Time to BT	Number of Biopsies	Mean PMN13F6 (%)	Mean PMN7C3 (%)	Mean PMN8C7 (%)
Remain in chronic phase				
4 mo-106 mo (median, 30)	16	82 $\pm$ 9	62 $\pm$ 14	68 $\pm$ 14
13 mo-49 mo Prior to BT (median, 23)	11	81 $\pm$ 12	71 $\pm$ 5	64 $\pm$ 15
2 mo-12 mo (median, 5.5)	8	68 $\pm$ 15	51 $\pm$ 17	46 $\pm$ 18
Blast crisis without extramedullary	11	14 $\pm$ 12	11 $\pm$ 10	6 $\pm$ 6
Blast crisis with extramedullary	12	20 $\pm$ 25	19 $\pm$ 25	13 $\pm$ 25

Abbreviation: BT, blast transformation.

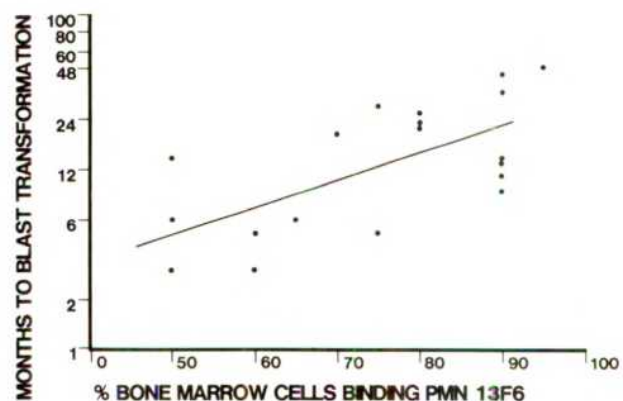


Fig 7. A scatter diagram indicating the relationship between the percentage of bone marrow cells expressing the PMN13F6 antigen and time from bone marrow biopsy to blast transformation. Time is plotted as $\log_{10}$. Only bone marrow biopsy specimens obtained more than 2 months prior to blast transformation are included in this analysis to determine the prognostic value of antigen expression for the prediction of blast transformation. The solid line represents conventional linear regression. Regression and correlation analysis determined that the correlation coefficient was $r = .6533$, which is significant at $P < .005$.

The appearance of increased numbers of myeloblasts and promyelocytes in the peripheral blood or bone marrow is often used as a sign of impending blastic crisis. Immature cells in the peripheral blood, used as a single sign, identified 27% of patients who entered the terminal phase within 6 months.¹³ Marked oscillations in the number of peripheral early myeloid cells limit the usefulness of this criterion.¹⁴ Since changes in the bone marrow may antecede those in the peripheral blood, bone marrow may be a better reflection of disease status. In our study, the relationship between the percentage of immature cells and the time to blastic crisis was significant ($P < .005$) and similar to the relationship between the percentage of antigen-positive cells and the time to transformation. Both sets of observations, however, were based upon a group of patients evaluated retrospectively and are therefore limited. Predictions based upon changes within a group, particularly one laboratory parameter, may not reflect individual outcomes. Thus, currently there is no universally accepted method for predicting the timing of blast transformation, and additional criteria are needed to provide predictive indicators.

Antigen expression on blood cells from patients with CML have been examined at the time of blastic transformation.³³⁻³⁶ Gallin et al, in association with our laboratory, previously noted that loss of an antigen present on the majority of mature peripheral neutrophils is associated with progression to the blastic phase of CML.³⁵ In that study, PMN7C3, one of the antibodies also used in this study, had variability of expression in neutrophils from CML patients at the time of blast crisis, but a severe deficiency of PMN7C3 was not noted. The difference between these findings and the current study is presumably due to analysis of peripheral cells, which are more mature, v bone marrow cells, which are more immature.

Griffin and coworkers, using a panel of monoclonal antibodies that recognize differentiation antigens of normal myeloid, erythroid, megakaryocytoid, and lymphoid cells, suggested that determination of antigenic phenotypes at blast crisis may provide clinically useful information for CML treatment protocols.³⁶ In that study leukemic cells from two thirds of 30 CML patients in blast crisis expressed no myeloid antigens when probed with a panel of monoclonal antibodies. Leukemic cells from the remaining one third of patients expressed an antigen associated with myeloid precursor cells.³⁷ Only one patient's cells also strongly expressed antigens found on more mature myeloid cells. These data are in agreement with our data that clearly show a deficiency in expression of some myeloid antigens at the time of blastic transformation.

We have not directly compared PMN13F6, PMN7C3, and PMN8C7 monoclonal antibodies with all other antibodies that have been used to investigate myeloid antigen expression in CML cells. However, PMN13F6 and PMN8C7 appear to be unique antibodies that recognize antigens with biochemical characteristics that have not previously been described. PMN7C3, which has been described in detail previously, binds to the C3bi receptor complex.

The present study demonstrates that there is a highly

significant loss of certain myeloid antigens on bone marrow cells from patients with CML at the time of blastic transformation compared with bone marrow cells examined at the time of diagnosis or during the stable phase. Furthermore, this loss appears to be a progressive loss with a significant correlation between expression of some myeloid antigens and time until development of blastic transformation. The data indicate that the loss of myeloid antigen expression may extend over many months rather than major antigenic changes that occur only at the time of blast crisis.

The progressive loss of myeloid antigen expression in CML is not strictly a function of lineage specificity. Antigen loss occurred in both myeloid (TdT-negative) and lymphoid (TdT-positive) blast transformation. The loss of myeloid antigen expression is also not strictly a function of differentiation specificity. Mature cells, in some cases, failed to express the antigens detected by these antibodies. PMN8C7 identifies an antigen that first appears at the metamyelocyte stage of differentiation and persists throughout maturation. There should be a significant relationship between the loss of PMN8C7 reactivity and morphology if progressive loss of this antigen were a function of increased numbers of immature cells. However, there was no correlation between the percentage of cells that expressed or failed to express the PMN8C7 antigen and the percentage of immature cells ($P < .20$). Even more intriguing is the highly significant, inverse relationship between the percentage of cells positive for PMN13F6, which identifies an antigen present throughout differentiation, and the percentage of immature cells. If antigen expression were simply a reflection of morphology, the percentage of PMN13F6-positive cells should remain constant or increase in parallel to the percentage of myeloid cells, regardless of the stage of differentiation.

Despite these observations and the suggested conclusions, speculation is warranted that lineage specificity, differentiation specificity, or both may be involved to some extent in the process of antigenic loss in CML. The clonal origin of the malignancy in CML has been well documented,¹ and it is known that the cell of origin is an early hematopoietic progenitor that is capable of giving rise to not only myeloid cells but also erythroid cells, megakaryocytes, monocytes, and lymphocytes. By conventional morphology and cytochemical analysis, the malignant cells present at blast transformation are recognized as myeloid blasts in 50% to 60% of the cases.¹⁵ Lymphoblast morphology occurs at the time of blast transformation in about one third of patients, usually involving both B cell lineage,^{38,39} and T cell lineage.⁴⁰⁻⁴² In addition, megakaryoblastic and monoblastic blast transformations are known to occur,^{33,43} and it has been suggested that erythroid blast crisis may occur much more frequently than previously recognized.⁴⁴ Thus, myeloid markers may be lost as the disease progresses due to the emergence of increased numbers of cells that are of nonmyeloid lineages. With improved methods of detection, features associated with two or more lineages have been shown to occur during blast transformation.^{35,36,39,40,44-46}

CML blast cells may represent an earlier or undifferentiated stage of maturation compared with the level of differ-

entiation at which a particular antigen is normally expressed. CML blasts may not express PMN13F6 antigen because the blasts are at a stage of differentiation preceding the normal appearance of this myeloblast antigen. Loss of the antigen detected by PMN13F6 was associated with the emergence of increased numbers of immature cells, but these cells were at a morphological level of differentiation at which there is expression of the antigen normally. Thus, antigenic expression during differentiation in CML cells is incongruent with morphological maturation. CML cells, which may appear similar to their normal counterparts at the same level of differentiation, clearly have abnormal antigenic patterns due to either selective expression or lack of expression of some enzymes or surface markers. Thus the highly significant, inverse relation between the percentage of cells expressing the antigen identified by PMN13F6 and the percentage of immature cells may be due to the emergence of abnormal immature cells, the identification of which is made easier by antigenic analysis.

In our study we did not separate the effects of expansion of nonmyeloid lineages, increases in blast cells, or selective loss of markers as a cause of antigen loss. The decision to ignore cell type in relation to antigen expression followed extensive analysis of all marrows with respect to various populations of cells and their contribution to overall cellularity. The myeloid elements and/or blast cells constituted the majority of the total marrow population in all CML biopsy specimens examined, and differential evaluation of nonmyeloid and nonblastic elements did not appear to contribute significantly to the understanding of antigen expression. Determination of antigen expression as the total percentage of cells that were positive for PMN13F6, PMN7C3, or PMN8C7 binding regardless of whether the cells appeared to be blastic, more

mature myeloid, or nonmyeloid in morphology may have enhanced the ability to demonstrate a correlation of antigen loss to blast transformation over a period of time. In addition, the percentage of the entire cell population that expresses or fails to express myeloid antigens in combination with morphological analysis of the percentage of immature v mature cells may provide better prognostic indicators than either variable alone.

The ability to determine the probability of blast transformation occurring within a certain time period would aid in deciding whether to delay or proceed with bone marrow transplantation. The increasingly optimistic results obtained with bone marrow transplantation justify additional studies regarding the use of antigen markers as indicators of the timing of blast transformation. In addition, the reexpression of normal neutrophil antigens after treatment-induced remissions suggests that monitoring antigenic expression may be useful in assessing response to therapy. The ability to predict impending or early blastic transformation during the chronic stage would provide a method to identify those patients who are at high risk of death and who would therefore be suitable candidates for earlier aggressive therapy prior to the development of overt blast transformation or for new, unproved therapeutic protocols. A prospective study with a larger number of patients and bone marrow biopsy samples performed at predetermined regular intervals is necessary to confirm the usefulness of myeloid antigen expression in combination with other indicators.

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Low Molecular Weight B Cell Growth Factor Induces Proliferation of Human B Cell Precursor Acute Lymphoblastic Leukemias

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Experiments were conducted to determine the effect of low mol wt B cell growth factor (L-BCGF) on B cell precursor acute lymphoblastic leukemia (ALL). L-BCGF induced a significant increase in ^3H -TdR incorporation in 28 of 37 bone marrow aspirates from patients with B cell precursor ALL, with stimulation indices ranging from 2 to 129. Fluorescence-activated cell sorting confirmed that in five of seven patients the common acute lymphoblastic leukemia antigen (CALLA)/CD10 positive leukemic cells

were responding directly to L-BCGF. L-BCGF was capable of inducing, in some patients, an increase in absolute viable cells and could also induce colony formation in vitro. The response of B cell precursor ALL was not attributable to β IL 1, IL 2, or γ interferon. These results indicate that the majority of B cell precursor ALL undergo a proliferative response to L-BCGF, suggesting a regulatory role for this lymphokine in the growth of B cell precursors.

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OVER THE PAST several years a number of lymphokines have been described that influence the proliferation and differentiation of human and murine B cells at various stages of development.¹⁻³ In humans these include low mol wt B cell growth factors (L-BCGF),^{4,5} high mol wt BCGF,⁶ B cell stimulatory factor 1 (BSF-1)/interleukin 4 (IL 4),⁷ B cell differentiation factor (BSF-2),⁸ B cell activation factor,⁹ pre-B cell growth factor,¹⁰ B cell inhibitory factor,¹¹ interleukin 2 (IL 2),¹²⁻²³ and gamma interferon.²⁴⁻²⁶ Analysis of the biochemical and structural characteristics of these lymphokines, their role in B cell activation, and the characterization of their target cells is currently an area of active investigation.

The 12 kd human L-BCGF is a T cell-derived lymphokine that acts on preactivated B cells, ie, activated with anti-IgM antibody.²⁷ L-BCGF is probably derived from a 60-kd cytosolic precursor protein,²⁸ and L-BCGF has recently been purified to a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.²⁹ Studies on L-BCGF-dependent B cell lines and activated B cells show the presence of $\sim 10^4$ high affinity and $\sim 3.5 \times 10^4$ low affinity receptors.³⁰ In experiments using neoplastic B cells, Ford et al³² have demonstrated a direct proliferative effect of L-BCGF on hairy cells³¹ and on cells from different B cell non-Hodgkins' lymphomas.

The factors influencing the proliferation of malignant

human B cell precursors are poorly understood. Experiments using colony assays for growth of B cell precursor acute lymphoblastic leukemias (ALL) suggest a role of soluble growth factors either provided by leukocyte feeder cells³³ or present in leukocyte-conditioned medium,^{34,35} and Töuw et al³⁴ suggested that one of the factors was IL 2. As part of an effort to examine factors influencing the growth of B cell precursor ALL (also termed "common" ALL or non-T/non-B ALL), we herein report on experiments using L-BCGF. Our results indicate that the majority of B cell precursor ALL undergo a proliferative response to this lymphokine.

MATERIALS AND METHODS

Leukemic specimens. Bone marrow aspirates from 37 patients with newly diagnosed B cell precursor ALL were analyzed. All specimens were obtained through the Cell Marker Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota. Only aspirates with >90% blasts were included. Diagnosis of B cell precursor ALL was based on standard morphological, cytochemical, and immunologic criteria. All samples expressed at least two of the three B cell-associated antigens p24/CD9,³⁶ common acute lymphoblastic leukemia antigen (CALLA)/CD10,³⁷ and gp42/CD24.³⁸ No sample contained more than 5% T cells based on expression of CD5³⁹ and CD7⁴⁰; more than 5% normal, mature B cells based on expression of surface Ig; more than 5% myeloid cells based on expression of CD13⁴¹; or more than 5% monocytes based on expression of CD11.⁴² Eight of the 37 bone marrow aspirates had been cryopreserved in liquid nitrogen for two to 24 months prior to analysis. We have analyzed the influence of freezing and thawing on the response to L-BCGF by comparing the L-BCGF response of seven noncryopreserved specimens to the L-BCGF response after having frozen and thawed the same specimens. Only samples with greater than 85% viable cells after thawing were analyzed, and no significant differences in the response to L-BCGF were observed (data not shown).

Proliferation assay. Bone marrow aspirates were separated on Ficoll-Hypaque (FH) gradients. Two $\times 10^5$ interface cells were incubated in 96 well flat-bottom plates (3596, Costar) in RPMI 1640 containing 10% vol/vol fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY), in the presence or absence of various concentrations of L-BCGF (Cellular Products Inc, Buffalo, NY). All experiments were set up in triplicate. Cells were harvested after 90 to 96 hours, except in the time course experiments.

Cell proliferation was determined by:

1. ^3H -TdR incorporation: ^3H -TdR (Amersham, Arlington Heights, IL; specific activity-20 Ci/mmol) was diluted in RPMI 1640/10% FBS and added at a

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concentration of 1 μ Ci per well for the last eight (patients 9 to 37 in Table 1) or 16 hours (patients 1 to 8). The cells were harvested on glass fiber filters using a cell harvester (M-24, Brandel, Gaithersburg, MD). The filters were dried for at least four hours, and ^{3} H-TdR incorporation was measured by liquid scintillation spectrometry. Stimulation indices are presented as the ratio of ^{3} H-TdR incorporated into cells incubated in the presence of L-BCGF compared to ^{3} H-TdR incorporated into cells incubated with no L-BCGF. We compared the results of 2-, 4-, 8-, and 16-hour incubations with ^{3} H-TdR. As no significant differences in the stimulation indices were observed between the eight- and the sixteen-hour incubations, we used the eight-hour incubation beginning with patient 9 (Table 1).

Table 1. Response of B Cell Precursor ALL Bone Marrow Aspirates to L-BCGF*

Patient No.	10% L-BCGF	No L-BCGF	Stimulation Index†
1	264 $\pm$ 117‡	95 $\pm$ 72	3 NS
2	145 $\pm$ 23	158 $\pm$ 51	1
3	101 $\pm$ 41	88 $\pm$ 14	1
4	138 $\pm$ 92	140 $\pm$ 76	1
5	98 $\pm$ 34	86 $\pm$ 70	1
6	2,308 $\pm$ 4	1,447 $\pm$ 192	2
7	2,270 $\pm$ 377	773 $\pm$ 3	3
8	7,052 $\pm$ 336	401 $\pm$ 116	14
9	10,618 $\pm$ 608	4,115 $\pm$ 456	3
10	12,569 $\pm$ 1,549	7,183 $\pm$ 775	2
11	22,991 $\pm$ 2,908	363 $\pm$ 121	63
12	16,708 $\pm$ 496	8,868 $\pm$ 251	2
13	24,172 $\pm$ 4,646	1,435 $\pm$ 148	17
14	17,024 $\pm$ 1,324	295 $\pm$ 126	58
15	853 $\pm$ 253	149 $\pm$ 91	6
16	5,725 $\pm$ 371	449 $\pm$ 97	13
17	2,780 $\pm$ 259	840 $\pm$ 62	3
18	9,622 $\pm$ 563	311 $\pm$ 21	32
19	55,242 $\pm$ 371	11,848 $\pm$ 820	5
20	18,962 $\pm$ 908	1,514 $\pm$ 69	13
21	1,562 $\pm$ 517	196 $\pm$ 22	8
22	711 $\pm$ 179	118 $\pm$ 34	6
23	8,330 $\pm$ 444	6,725 $\pm$ 156	1
24	1,714 $\pm$ 24	1,335 $\pm$ 73	1
25	626 $\pm$ 107	329 $\pm$ 75	2
26	130 $\pm$ 48	126 $\pm$ 25	1
27	4,788 $\pm$ 281	409 $\pm$ 4	12
28	9,799 $\pm$ 475	341 $\pm$ 223	29
29	401 $\pm$ 120	87 $\pm$ 11	5
30	17,850 $\pm$ 1,035	289 $\pm$ 56	62
31	306 $\pm$ 283	350 $\pm$ 30	1
32	491 $\pm$ 23	205 $\pm$ 42	2
33	3,473 $\pm$ 1,114	163 $\pm$ 76	21
34	15,654 $\pm$ 965	121 $\pm$ 2	129
35	718 $\pm$ 15	116 $\pm$ 13	6
36	40,075 $\pm$ 5,674	14,406 $\pm$ 280	3
37	206,820 $\pm$ 21,197	22,181 $\pm$ 3,263	9

*Leukemic cells were cultured in the presence or absence of 10% vol/vol L-BCGF for 96 hours and were pulsed with ^{3} H-TdR for the final eight (patients 9 to 37) or 16 (patients 1 to 8) hours.

†Stimulation index: Ratio of ^{3} H-TdR incorporation in the L-BCGF-treated cells compared to controls treated with no L-BCGF.

‡Mean cpm $\pm$ SD of ^{3} H-TdR incorporation in triplicate cultures.

2. Cell count: Cells from triplicate wells were vigorously pipetted and pooled. The wells were checked under an inverted microscope to ensure that all cells had been removed. Cell viability and absolute cell counts were analyzed by trypan blue exclusion.
3. Colony assay: One $\times 10^5$ leukemic bone marrow cells were suspended in 1 mL of RPMI 1640 containing 25% FBS, 5×10^{-5} mol/L 2-mercaptoethanol, 0.9% methylcellulose in the presence or absence of 10% vol/vol L-BCGF. The cells were cultured in 35-mm petri dishes at 37°C in a humidified 5% CO₂ atmosphere and analyzed by inverted microscopy after ten days.

Lymphokines. Partially purified L-BCGF was obtained from Cytokine Technology, Buffalo, NY. L-BCGF did not contain detectable IL 2 activity based on the inability to support the growth of IL 2-dependent CTLL-20 cells (assay generously conducted by Dr Siew-Lin Wee, Immunobiology Research Center, University of Minnesota). Highly purified L-BCGF was isolated as previously described.³⁰ Recombinant beta interleukin 1 (β IL 1), recombinant interleukin 2 (IL 2), and recombinant gamma interferon (gamma IF) were purchased from Genzyme Corporation, Boston.

Sorting of CALLA/CD10 positive cells. Bone marrow aspirates were used for cell sorting. In seven of eight samples the cells had been cryopreserved until the results of the effect of L-BCGF on the fresh bone marrow aspirates were known. The cells were thawed from liquid nitrogen, washed twice in fluorescence buffer (PBS containing 2.5% FBS and 0.02% sodium azide), stained with the anti-CALLA/CD10 monoclonal antibody (MoAb) BA-3 for 30 minutes at 4°C, washed once in fluorescence buffer, stained with fluorescein isothiocyanate (FITC) goat antimouse Ig (Cappel Worthington Biochemicals, Malvern, PA, dilution 1:20) for 30 minutes, and washed three times. Three- 10×10^6 CALLA/CD10 positive cells were then sorted on a FACS IV (Becton Dickinson, Mountain View, CA). Forward and 90° light-angle scatter were used to gate on the viable leukemic cells. Only cells exhibiting fluorescence intensity clearly above background were sorted into the CALLA/CD10 positive fraction. These cells constituted 85% to 90% of the leukemic cell population. To exclude the potential influence of BA-3 on the in vitro L-BCGF response, we incubated and stained cells with BA-3 and FITC goat antimouse Ig, but did not sort them.

Statistics. For analysis of the differences between L-BCGF stimulated and unstimulated cells we used the student's *t* test on a log scale. Results with *P* < 0.05 were considered statistically significant.

RESULTS

Response of B cell precursor ALL bone marrow aspirates to L-BCGF. We initially analyzed the effect of L-BCGF on the incorporation of ^{3} H-TdR in FH-isolated cells from bone marrow aspirates of 37 patients with B cell precursor ALL. The results obtained when cells were stimulated with 10% vol/vol L-BCGF are presented in Table 1. In 28 of the 37 patients (74%) a significant increase in ^{3} H-TdR incorporation was observed. The individual responses varied widely, with stimulation indices ranging from 2 to 129 and 12 of the patients having a stimulation index >10.

In 15 of 17 responding patients the effect of different concentrations of L-BCGF was studied. The results shown in Fig 1 underscore the variable sensitivity to L-BCGF. Cells from 5 of 15 patients showed a significant increase of ^{3} H-TdR incorporation over background in the presence of only 1% vol/vol L-BCGF. Cells from four patients required

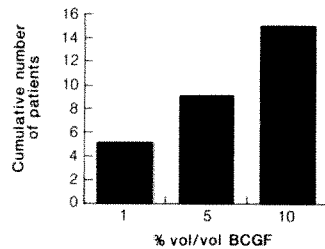


Fig 1. Variability in response of FH-isolated bone marrow cells from 15 patients with B cell precursor ALL who responded to L-BCGF. Cells were incubated with increasing concentrations of L-BCGF or no L-BCGF for 96 hours and pulsed with ^3H -TdR for the final eight or 16 hours. Data show the cumulative number of patients that, at a given concentration of L-BCGF, exhibited a statistically significant increase ($P < 0.05$) in ^3H -TdR incorporation compared to the controls, as determined by the student's t test.

5% vol/vol L-BCGF, and the remaining six patients required 10% vol/vol L-BCGF to show a response. CALLA/CD10 positive cells were sorted from three patients, and the L-BCGF response of the sorted cells paralleled the results obtained with unsorted cells (data not shown). L-BCGF concentrations of $>15\%$ vol/vol inhibited ^3H -TdR incorporation in B cell precursor ALL.

Response of CALLA/CD10 positive leukemic cells to L-BCGF. To confirm that the L-BCGF response was not due to ^3H -TdR incorporation by nonleukemic cells, we isolated CALLA/CD10 positive leukemic cells from seven patients by cell sorting and examined their response to L-BCGF. We chose this approach, since CALLA/CD10 is not expressed on surface Ig positive normal B cells, and $>90\%$ of cells in these aspirates were CALLA/CD10 positive. Cells stained with BA-3 and FITC goat antimouse Ig, but not sorted, were used to control for the effects of surface-bound antibody on L-BCGF-induced ^3H -TdR incorporation. The results in Table 2 indicate that in five of seven patients (Nos. 7, 9, 13, 15, and 18), the L-BCGF response of CALLA/CD10 positive cells essentially recapitulated the L-BCGF response of stained, unsorted cells. In patient No. 17 a twofold increase in ^3H -TdR incorporation was observed, but this was not statistically significant. In patient No. 8 a comparison of stained, unsorted cells (Table 2) with cells not stained with BA-3 and FITC goat antimouse Ig (Table 1)

clearly shows that leukemic cell-bound antibody did not influence the response to L-BCGF. Failure of this patient's CALLA/CD10 positive leukemic cells to respond could be attributable to (1) cell damage during the sorting procedure, (2) ^3H -TdR incorporation by surface Ig positive, CALLA/CD10 negative normal B cells in the bone marrow aspirate, or (3) dependency on accessory cells such as monocytes or T cells. In patient No. 7 the L-BCGF response of CALLA/CD10 positive cells was $\sim$ tenfold higher than in the stained, unsorted bone marrow aspirate, suggesting an inhibitory effect mediated by factors produced by contaminating normal cells. To formally exclude this as an explanation for the nonresponding leukemic samples, we sorted CALLA/CD10 positive cells from one nonresponding patient (No. 1 in Table 1) and examined their response to L-BCGF. These sorted CALLA/CD10 positive cells did not respond to L-BCGF (Table 2).

Cell proliferation studies. Time sequence studies of ^3H -TdR incorporation and absolute viable cell counts of the CALLA/CD10 positive leukemic cells were conducted on several patients. In the first patient (Fig 2A, patient No. 7 in Table 1) an increased incorporation of ^3H -TdR was seen after day 2 and continued through day 6 in the presence of L-BCGF. The cell count in the L-BCGF-treated cultures dropped over the first three days to $300 \times 10^3/\text{mL}$ on day 3 and then increased slightly to $400 \times 10^3/\text{mL}$ on day 6 (Fig 2B). The cell count in the control wells plateaued at $150 \times 10^3/\text{mL}$ from days 3 to 6. In the second patient (Fig 2C, No. 18 in Table 1) ^3H -TdR incorporation steadily increased from day 2 to day 6 in the L-BCGF-treated cultures. The cell number remained stable from day 2 to day 6 in the presence of L-BCGF, whereas the cell number in the controls dropped to 10% of the starting concentration by day 6 (Fig 2D). In the third patient (Fig 2E, No. 15 in Table 1) an increase in ^3H -TdR incorporation in L-BCGF-treated cultures compared to controls was detectable on day 1 and peaked on day 3. The initial cell loss was substantial in the L-BCGF-treated cultures, dropping to $260 \times 10^3/\text{mL}$ within the first 24 hours followed by an increase to $530 \times 10^3/\text{mL}$ by day 3 (Fig 2F). In a fourth patient (No. 37 in Table 1) we analyzed absolute viable cell numbers over a longer period of time (Fig 3). This patient's leukemic cells underwent a rigorous proliferative response to L-BCGF, with the absolute

Table 2. L-BCGF Response of CALLA/CD10 Positive Leukemic Cells*

Patient No.	CALLA/CD10 Positive Cells			Stained/Unsorted Cells		
	10% L-BCGF	No L-BCGF	Stimulation Index†	10% L-BCGF	No L-BCGF	Stimulation Index
7	23,911 $\pm$ 3,258‡	2,497 $\pm$ 291	10	2,181 $\pm$ 195	811 $\pm$ 146	3
8	190 $\pm$ 77	111 $\pm$ 40	2 NS	6,698 $\pm$ 701	470 $\pm$ 138	14
9	6,212 $\pm$ 208	3,362 $\pm$ 168	2	8,335 $\pm$ 143	3,020 $\pm$ 1,347	3
13	21,072 $\pm$ 1,749	839 $\pm$ 47	25	22,843 $\pm$ 1,410	1,370 $\pm$ 76	17
15	5,092 $\pm$ 558	136 $\pm$ 59	37	2,168 $\pm$ 520	96 $\pm$ 16	23
17	599 $\pm$ 216	259 $\pm$ 11	2 NS	2,348 $\pm$ 208	880 $\pm$ 167	3
18	3,166 $\pm$ 145	95 $\pm$ 3	33	8,830 $\pm$ 420	2,954 $\pm$ 118	30
1	116 $\pm$ 16	92 $\pm$ 16	1	188 $\pm$ 103	270 $\pm$ 185	1

*CALLA/CD10 positive cells isolated by cell sorting, or stained/unsorted cells were cultured in the presence or absence of 10% vol/vol L-BCGF for 96 hours and were pulsed with ^3H -TdR for the final eight to 16 hours.

†See Table 1.

‡See Table 1.

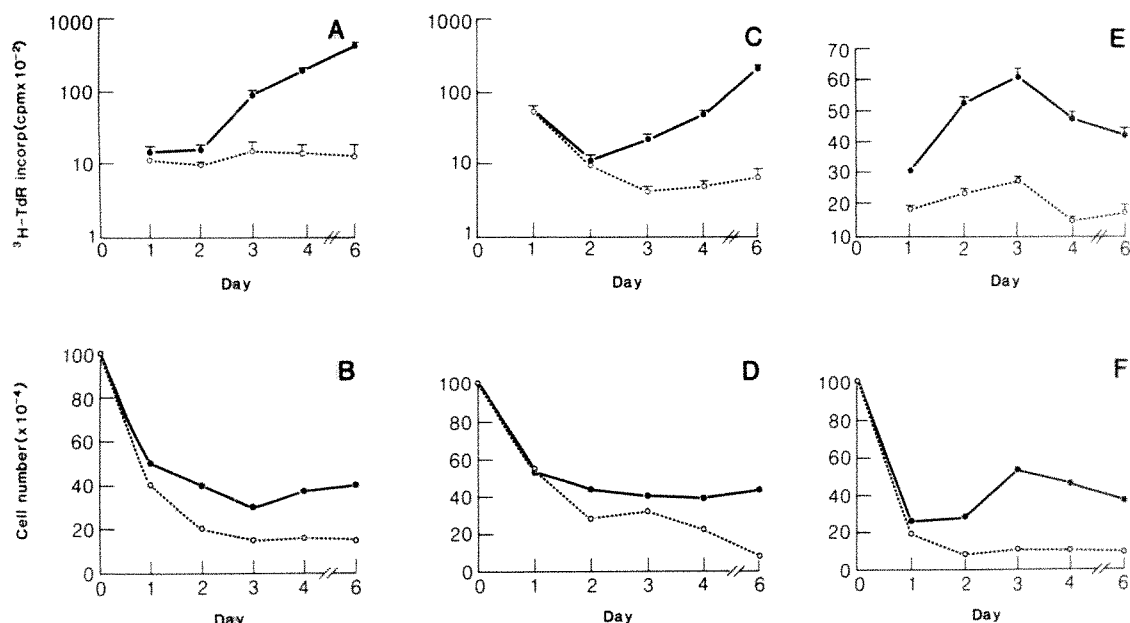


Fig 2. Time sequence of $^3\text{H-TdR}$ incorporation (A, C, E) and absolute viable cell numbers as determined by trypan blue exclusion (B, D, F) of CALLA/CD10-positive leukemic cells after incubation in 10% vol/vol L-BCGF (—●—) for zero to six days. Control cultures (---○---) did not contain L-BCGF. The $^3\text{H-TdR}$ incorporation data points represent the mean of triplicate values ± 1 SD (horizontal bars). The absolute cell number data points were derived by pooling triplicate wells and dividing the total viable cell count by three. A and B are patient No. 7; C and D are patient No. 18; and E and F are patient No. 15 from Table 1. Note that the ordinates in A and C are on a log scale.

viable cell number doubling at five days and tripling at ten days, compared to day 0. Note that control cells cultured with no L-BCGF dropped in absolute viable cell number.

We also used a colony assay to examine the *proliferative* response to L-BCGF. When cells from patient No. 36 (Table 1) were plated in the presence of L-BCGF, dramatic colony formation was observed by day 10 (Fig 4A), whereas no colony formation was observed in cells cultured without L-BCGF (Fig 4B). The cloning efficiency for the L-BCGF cultures was 8.3% for cluster formation (>10 cells) and 0.4% for colony formation (>50 cells). The phenotype of the colony-forming cells was identical to the original, uncultured cells ($\text{CD}9^+$, $\text{CD}10^+$, $\text{CD}24^+$, and cytoplasmic and surface IgM^-).

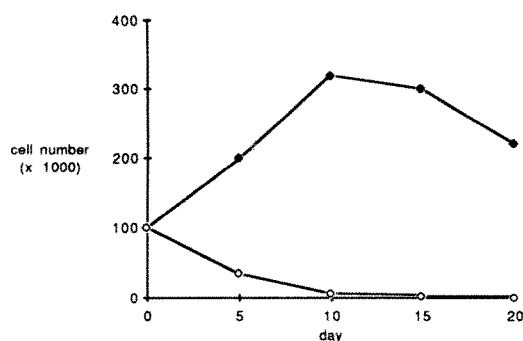


Fig 3. Cell growth of B cell precursor ALL (No. 37 in Table 1) in response to L-BCGF. Cells were incubated in the presence (—●—) or absence (---○---) of 10% vol/vol L-BCGF and quantitated at different time points. Cell viability was assessed by trypan blue dye exclusion.

Response of B cell precursor ALL to highly purified L-BCGF and other recombinant lymphokines. To confirm that the response of B cell precursor ALL was not attributable to a contaminant in the partially purified commercial product, we compared the response of a B cell precursor ALL to partially purified and highly purified L-BCGF. Leukemic cells from patient No. 36 (Table 1) had been maintained in partially purified L-BCGF for 20 days, followed by L-BCGF-free medium for 25 days. Under L-BCGF-free conditions the cells remained viable and showed little change in concentration. Results in Table 3 show the response of these cells to the two sources of L-BCGF. It is very apparent that 0.1% vol/vol and 1.0% vol/vol *highly purified* L-BCGF-triggered $^3\text{H-TdR}$ incorporation in the leukemic cells, comparable to the partially purified L-BCGF. Since units of activity are not defined in these preparations, it is not possible to directly equate them. Higher concentrations of highly purified L-BCGF (10% vol/vol) inhibited $^3\text{H-TdR}$ incorporation.

We examined the $^3\text{H-TdR}$ incorporation of B cell precursor ALL to increasing concentrations of $\beta\text{IL 1}$, IL 2, gamma IF, and L-BCGF. Whereas the leukemic cells exhibited a dose-dependent response to partially purified L-BCGF, no response occurred to $\beta\text{IL 1}$ (0.1 to 10 units/mL), IL 2 (10 to 1,000 units/mL), or gamma IF (10 to 10,000 units/mL; data not shown).

DISCUSSION

The growth factors and their receptors, which mediate the proliferation of B cell precursor ALL, are poorly understood. Prior studies reporting colony formation by B cell precursor

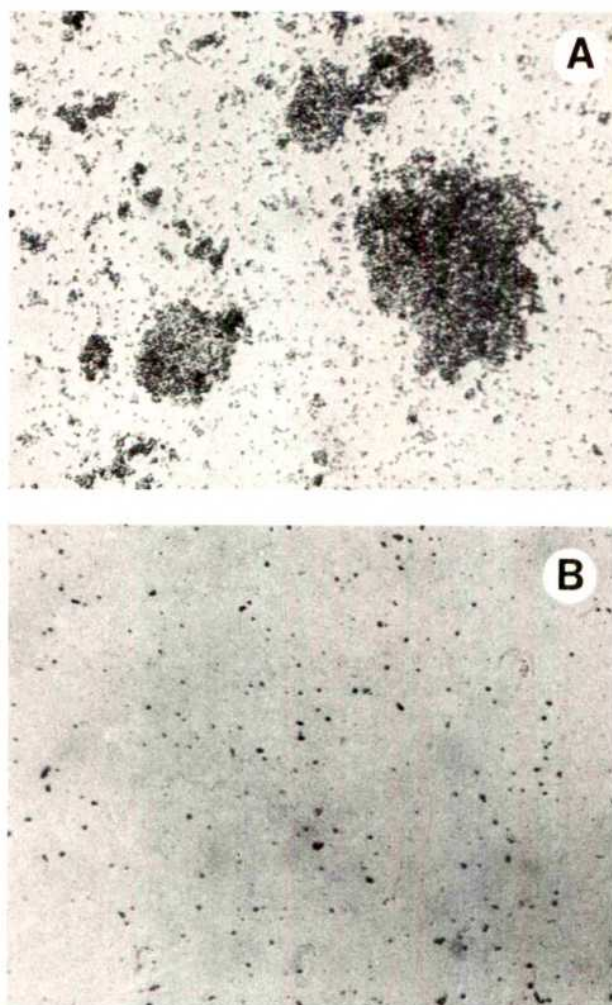


Fig 4. Colony formation of a B cell precursor ALL (No. 36 in Table 1) cultured in the presence (Fig 4A) or absence (Fig 4B) of 10% vol/vol L-BCGF for ten days. Cells from this patient were initially cultured in the presence of L-BCGF for 20 days. The cells were then split, and half of them were maintained in medium containing 25% fetal bovine serum (FBS) with no L-BCGF for 25 days. Under these conditions the cells remained viable and the cell number was stable. The phenotype prior to the colony formation experiment was unchanged as compared to the initial analysis (CD9 positive, CD10/CALLA positive, CD24 positive, negative for surface and cytoplasmic IgM). No colony formation was seen in the cultures without L-BCGF (Fig 4B).

ALL have shown a dependency on soluble factors provided by leukocyte feeders³³ or leukocyte-conditioned medium.^{34,35} Although the biochemical nature of the required growth factors were not elucidated, Touw et al³⁴ showed that B cell precursor ALL colony formation was enhanced by recombinant IL 2 in some cases.

In the present study we have provided evidence that ~75% of B cell precursor ALL respond to L-BCGF. Examination of Table 1 shows that L-BCGF-cultured cells from 28 of 37 patients underwent a significant increase in ³H-TdR incorporation compared to control cells cultured without L-BCGF. Cell sorting experiments confirmed that, in most cases, the CALLA/CD10 positive leukemic cells were responding

Table 3. Comparison of the B Cell Precursor ALL Response to Partially and Highly Purified L-BCGF

Culture Conditions		Response*	Stimulation Index†
No L-BCGF		3,872 ± 69‡	—
Highly purified L-BCGF	0.1%	8,713 ± 1,434	2
	1.0%	29,277 ± 6,065	8
Partially purified L-BCGF	5.0%	14,400 ± 67	4
	10.0%	35,046 ± 2,102	9

*Leukemic cells from patient No. 36 (Table 1) had been maintained in partially purified (Cytokine Technology) L-BCGF for 20 days, followed by L-BCGF-free medium for 25 days. The cells were then cultured with various concentrations of partially or highly purified L-BCGF for 96 hours and were pulsed with ³H-TdR for the final eight hours.

†See Table 1.

‡See Table 1.

directly to L-BCGF (Table 2), although exceptions were noted (patient No. 8). The L-BCGF response of CALLA/CD10 positive cells from patient to patient showed broad variation (Table 2), and explanations for this difference are discussed below. A critical experiment in this study was the direct comparison of the B cell precursor ALL response to partially purified (Cytokine Technology) and highly purified L-BCGF.²⁹ Data in Table 3 show that the proliferative response of B cell precursor ALL to the two L-BCGF sources was essentially the same. A second critical experiment was the demonstration that L-BCGF promoted colony formation of fresh B cell precursor ALL in vitro (Fig 4A). Increases in absolute cell numbers were also demonstrated in suspension cultures (Fig 2), although the responses were variable. Some leukemic specimens (eg, Figs 2C, 2D) showed an increase in ³H-TdR incorporation with no increase in absolute viable cell number. This suggests that the dividing cells may only be compensating for the cells dying in culture, and L-BCGF is not able to numerically expand these cells in vitro.

Several variables could be invoked to explain the patient-to-patient variability in response to L-BCGF, including: (1) differences in L-BCGF receptor expression/affinity that may reflect distinct stages of development at which a given leukemia is arrested, (2) dependency on external growth factors other than (or in addition to) L-BCGF, (3) dependency on accessory cells such as monocytes or T cells, and (4) dependency on an autocrine growth mechanism. Accessory cell dependency seems unlikely, since highly purified (>99%) CALLA/CD10 positive leukemic cells underwent a rigorous response to L-BCGF (Table 2). Dependency on other growth factors is a plausible explanation. One obvious candidate is IL 2, since this lymphokine has been shown by numerous laboratories to induce normal B cells to proliferate and/or differentiate.¹²⁻²³ Touw et al³⁴ showed that IL 2 enhanced B cell precursor ALL colony formation in the presence of leukocyte feeders. In contrast, we could find no role for IL 2 in the proliferation of B cell precursor ALL. Furthermore we studied 12 L-BCGF responders in Table 1 for their response to L-BCGF and 10 to 100 units/mL of IL 2 but found no evidence for a synergistic effect (data not shown). The discordance between our IL 2 results and those of Touw et al³⁴ might be attributable to the assay systems under study, ie, colony formation v ³H-TdR incorporation.

We feel that L-BCGF receptor expression may well explain the patient-to-patient variability observed in Tables 1 and 2. This variability could manifest itself at the quantitative level of L-BCGF receptor expression or at the level of signal transduction subsequent to binding of L-BCGF to its receptor. A resolution of these various possibilities awaits identification and characterization of the L-BCGF receptor.

In conclusion we have shown that the majority of B cell precursor ALL undergo a direct proliferative response to L-BCGF. We found no general role for IL 2 in B cell precursor ALL proliferation. Thus we believe L-BCGF may

be the predominant but not necessarily sole growth stimulus. The possibility that perturbation of L-BCGF/L-BCGF receptor interaction might disrupt growth of B cell precursor ALL should now be considered.

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Epitope Mapping of Functional Domains of Human Factor Va With Human and Murine Monoclonal Antibodies. Evidence for the Interaction of Heavy Chain With Factor Xa and Calcium

By Anjanayaki E. Annamalai, A. Koneti Rao, Hui Chong Chiu, Danny Wang, Asim K. Dutta-Roy, Peter N. Walsh, and Robert W. Colman

We have purified a unique neutralizing IgG₁, κ monoclonal antibody (MAb) against factor V (F-V) from a patient's plasma. This MAb (H2) demonstrated specificity for human F-V heavy chain (D), mol wt 105,000. Using an enzyme-linked immunosorbent assay (ELISA) we assessed the competitive binding to F-Va of H2, H1 (human MAb directed to light chain, F1F2), and two murine MAbs, B38 (to F1F2) and B10 (to activation peptide C1). All four antibodies are of high affinity with K_D varying from 0.17 to 1.17×10^{-10} mol/L. They recognized distinct epitopes in F-V. F-Xa competed in a concentration-dependent fashion for binding of H1, H2, and B38 but not B10 to F-V/Va in the absence of phospholipids or platelets. Thus both F1F2 and

D polypeptides of F-Va but not C1 interacted with F-Xa. All MAbs bound to F-V/Va in the absence of Ca^{++} . However, free Ca^{++} (0.1 to 4.0 mmol/L) increased the amount of H1 and H2 bound to factor V/Va, 1.65-fold and 3.65-fold, respectively but had little effect on the binding of either murine MAbs. Prothrombin (20 $\mu\text{g/mL}$ to 400 $\mu\text{g/mL}$) in the absence of phospholipid did not inhibit the binding of MAbs. These studies provide evidence for the first time for a direct interaction between human F-Va heavy chain and F-Xa and Ca^{++} and for the direct binding of F-Xa to F-Va in the absence of phospholipids or platelets and enhance our understanding of functional F-V domains.

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METHODS

Materials. Goat antirabbit IgG conjugated with alkaline phosphatase, paranitrophenyl phosphate, alkaline phosphatase, type VII-T, and protein A-Sepharose CL-4B were obtained from Sigma Chemical Co, St Louis. Human immunoglobulin subtyping kits were purchased from Miles Laboratories, Elkhart, IN. Reacti-Gel (6X) was purchased from Pierce Chemical Co, Dekalb, IL. Kodak X-Omat AR x-ray film was obtained from Eastman, Kodak Co, Rochester, NY; (p-Amidinophenyl)methanesulfonyl fluoride (p-APMSF) from Calbiochem, Behring Diagnostics, La Jolla, CA; and N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-arginine-p-nitroanilide hydrochloride (S-2222) from Kabi Vitrum AB, Helena Laboratories, Beaumont, TX. Ninety-six-well polystyrene Nunc-Immuno-plates were from Thomas Scientific Co, Swedesboro, NJ. Purified thrombin (3209 NIH U/mg) was a gift from Dr John Fenton, New York State Department of Health. All other chemicals were of reagent grade.

Human monoclonal antibody (H2) against human factor V. Blood was collected from the patient with acquired neutralizing antibody to factor V⁷ in 1:10 vol of 3.8% sodium citrate. The plasma was prepared by centrifuging blood for ten minutes at 2,000 g at room temperature and stored at -70°C . For purification of antibody the plasma was incubated at 56°C for 30 minutes to dissociate the antigen-antibody complexes.⁷ The IgG fraction was then precipitated with 45% saturated ammonium sulfate. The precipitate was dissolved in 0.02 mol/L Tris buffer, pH 7.2 containing 0.15 mol/L

HUMAN PLASMA factor V is a procofactor in the prothrombinase complex, which includes activated factor X, calcium and phospholipids, or platelets. When activated by thrombin, factor V (Va) accelerates the conversion of prothrombin to thrombin by several orders of magnitude. Mouse monoclonal antibodies (MAbs) against bovine¹ and human² factor V have been described. The formation of complexes composed of bovine factor V and MAbs has been demonstrated using light scattering and radioimmunoassay (RIA).¹ Two of the murine MAbs against bovine factor V have been shown to react respectively with the 94,000- and 74,000-dalton polypeptides that are noncovalently linked by Ca^{++} to form bovine factor Va.³ Foster et al² developed ten mouse MAbs against human factor V and demonstrated that one of them reacted with the light chain F1F2 (mol wt 71,000/74,000) of human factor Va. A naturally occurring human MAb antibody (H1) reported by Coots et al⁴ has been shown by Wilson et al⁵ to be specifically reactive against the light chain of human factor Va. We have recently characterized two murine MAbs against human factor V, B38, and B10 and demonstrated that they recognize epitopes on F1F2 and an activation peptide (mol wt 150,000) respectively in thrombin-cleaved factor V.⁶ To date no MAb has been reported specific to the heavy chain (D) of human factor Va, mol wt 105,000.

Recently we reported human MAbs circulating with human factor V as immune complexes in a patient.⁷ In this paper we describe the purification of a single class of immunoglobulin, IgG 1, with κ light chain containing factor V neutralizing antibody from the plasma of that patient and its characterization as specific for a site on the D polypeptide of factor Va. We then determined the relationship between the epitope of this antibody (H2) and other MAbs against human factor V developed (B38, B10) or purified (H1) in our laboratory. MAbs to specific epitopes should be useful probes to study the structure and function relationships of human factor V/Va. Therefore we studied the interaction of prothrombin and two components of prothrombinase complex (factor Xa and Ca^{++}) with these MAbs to characterize better the spatial arrangement of these components with respect to distinct sites in factor V/Va.

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NaCl (TBS), dialyzed at 4°C against the same buffer with two changes, and filtered through a 0.22 µm filter. The clarified solution was then dialyzed overnight at 4°C against citrate-phosphate buffer, pH 7.0 (0.1 mol/L citric acid was added to 0.2 mol/L Na₂HPO₄ until the desired pH was obtained). Chromatography of this sample on Protein A-Sepharose column was performed according to the procedure of Duhamel et al.⁸ The heavy chain subclass and the light chain type of IgG eluted from the column were determined by enzyme-linked immunosorbent assay (ELISA) using rabbit antibodies to human immunoglobulin subtypes and alkaline phosphatase-conjugated goat antirabbit IgG.

Human monoclonal antibody (H1) against factor V. Human plasma-containing inhibitory activity against factor V was a kind gift of Dr Helen Glueck, Cincinnati, OH and was purified as described by Chiu et al.⁹ H1 is IgG4, λ subtype, neutralizes factor V activity and is directed to factor Va light chain (F1F2).

Preparation and characterization of murine monoclonal antihuman factor V (B38 and B10). The murine MABs were prepared in our laboratory as described earlier¹⁰ and have been extensively characterized.⁶ The immunoglobulin type of each is IgG1 κ. B38 neutralizes factor V activity and is directed to the light chain (F1F2). B10 does not neutralize factor V activity but recognizes an epitope on the activation peptide (C1).

Purification of human plasma factor V. Factor V was purified by an immunoaffinity procedure⁹ adapted for a murine monoclonal antifactor V (B38) instead of a human MAB. The immunoabsorbent was prepared identically,⁹ but nonspecifically bound proteins were removed by washing with 0.02 mol/L Tris buffer, pH 7.2 containing 0.37 mol/L NaCl followed by a wash with the same buffer containing 5% glycerol. The specifically bound factor V was eluted with the Tris buffer containing 0.15 mol/L NaCl, 25% glycerol and 50% ethylene glycol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified factor V showed a single band corresponding to the mol wt of 330,000. The specific activity ranged from 70 to 100 U/mg and increased to 17- to 20-fold upon activation with thrombin.

Radiolabeling of human factor V. Purified factor V was labeled with ¹²⁵I by the iodogen method as modified by Tuszyński et al.¹¹ The radiolabeled factor V had a specific activity of 4.67×10^6 cpm/µg.

Thrombin activation of ¹²⁵I human factor V. Radiolabeled factor V (1 to 2 U/mL) was activated with thrombin (0.05 to 0.1 U/mL) as described by Annamalai et al.¹⁰

Epitope specificity of human monoclonal antihuman factor V (H2). Purified human monoclonal IgG was coupled to Reacti-Gel 6X. To remove acetone the suspension of Reacti-Gel (500 µL) was washed on a coarse-fritted glass-filter funnel with gentle suction and several bed volumes of ice cold water. The moist cake was transferred to 400 µL of 0.1 mol/L borate buffer, pH 8.0 in an Eppendorf tube containing 0.25 mg/mL human monoclonal IgG or normal human IgG (control). The mixtures were rotated end over end overnight at 4°C. At the end of incubation the gel-ligand mixtures were centrifuged at 12,000 g in a microcentrifuge for four minutes. The precipitates were washed with ice-cold 0.5 mol/L Tris buffer pH 8.0 two times and left overnight at 4°C in the same buffer with gentle mixing. The suspensions were centrifuged and resuspended in 0.075 mol/L Tris buffer pH 7.2 containing 0.075 mol/L NaCl, 20 mmol/L Ca⁺⁺, or 20 mmol/L EDTA and 0.1% bovine serum albumin (BSA). Radiolabeled factor Va, 0.15 µg (0.7×10^6 cpm) was added to these tubes and incubated overnight with mixing. Then the gel-ligand mixtures were transferred to disposable plastic columns with filter discs and washed successively with 10 mL each of the following buffers to remove all the unadsorbed and nonspecifically adsorbed materials from the immunoabsorbents: Buffer 1, 0.02 mol/L Tris pH 7.2, 0.15 mol/L NaCl, 0.1% BSA, 0.1% Tween-20, and 20 mmol/L Ca⁺⁺ or EDTA; Buffer 2, 0.02 mol/L Tris, pH 7.2, 0.5 mol/L NaCl, 0.1% BSA, and 20 mmol/L Ca⁺⁺ or EDTA;

Buffer 3, 0.02 mol/L Tris, pH 7.2, 0.15 mol/L NaCl, and 20 mmol/L Ca⁺⁺ or EDTA. The immunoabsorbents containing specifically bound factor Va polypeptide were transferred to Eppendorf tubes and centrifuged for four minutes. The pellets were suspended in 10% sodium dodecyl sulfate (SDS), boiled for ten minutes to dissociate the antigen, and centrifuged. The supernatants containing radiolabeled factor Va fragment then were electrophoresed.

SDS slab-PAGE and autoradiography. Electrophoresis was performed using 3% to 15% gradient polyacrylamide gel in a Tris buffer system according to the method of Laemmli.¹² Purified factor V, Va, fibrinogen, and mol wt standards consisting of myosin (200,000), phosphorylase B (97,400), BSA (68,000), Ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), and cytochrome C (12,300) were applied along with the samples. The gels were fixed and stained with Coomassie brilliant blue R for protein. After destaining the gels were dried and subjected to autoradiography with Kodak X-Omat AR x-ray film.

Conjugation of purified MABs to alkaline phosphatase. MABs were dialyzed against 0.02 mol/L Na phosphate containing 0.15 mol/L NaCl, pH 7.2 phosphate-buffered saline (PBS) overnight at 4°C. Alkaline phosphatase (921 units) and 25% glutaraldehyde (final concentration 0.2% vol/vol) were added to 0.2 mg of anti-V IgG in a total volume of 1.5 mL. The mixture was incubated for two hours at room temperature and then dialyzed against PBS overnight with two changes. The enzyme-antibody conjugate was further dialyzed against 0.05 mol/L Tris buffer, pH 8.0 containing 1 mmol/L MgCl₂ at 4°C with two changes. 1% BSA and 0.02% Na₂S₂O₃ were added to the conjugate and stored at 4°C.

Binding specificity of alkaline-phosphatase conjugated and unconjugated MABs to factor V/Va. Since epitope mapping was carried out using enzyme-conjugated and unconjugated antibodies, it was first necessary to determine whether conjugation affected the ability of the MABs to bind to factor V/Va. A competitive ELISA (cELISA) was employed to accomplish this goal and was performed as previously described⁹ using microtiter plates coated with purified factor V/Va. The plates were incubated overnight at 4°C with each of the conjugated and unconjugated antibodies, predetermined to give 70% saturation of the antigenic sites along with increasing concentrations of purified factor V/Va (0.095 nmol/L to 3.04 nmol/L). After extensive washing the amount of bound enzyme-antibody conjugate was detected by incubating with p-nitrophenyl phosphate. Controls without antigen but containing the anti-V antibody-enzyme conjugate and substrate or without the antibody-enzyme conjugate but containing the antigen and substrate were included to correct for the nonspecific binding. Each experiment was done in triplicate.

Dissociation constants (K_D) of purified MABs for their antigenic determinants. The dissociation constants of the purified murine and human MABs to human factor V/Va were determined by the method of Friguet et al.¹³ The absorbance at 405 nm resulting from the enzymatic activity of the immunoconjugate bound to the adsorbed factor V/Va v various concentrations of each of the purified MAB in ELISA was found to be linear. A concentration of each antibody that saturated 60% to 70% of the antigenic determinants of the adsorbed factor V/Va was then incubated with various concentrations of purified factor V/Va (0.57 nmol/L to 18.4 nmol/L) in equal volume overnight until equilibrium was reached. Free antibody concentration at equilibrium for each incubation mixture was determined by ELISA under the same conditions as was done initially for choosing the antibody concentration. Absorbance (A_o) measured in ELISA for the incubation mixture containing only the antibody without added antigen would be related to the total antibody concentration, (Ab)_t. For the total antibody concentration, (Ab)_t incubated with a given antigen concentration, at equilibrium the absorbance (A) measured in ELISA would be related to the free antibody concentration, (Ab)_f. The method is applicable even in the

case of unpurified antibodies. From a plot of $A_{500}/A_{500} - A_{500} \nu 1/(Ag)_t$, the K_D can be calculated from the slope.¹⁴

Epitope mapping of the four MAbs against factor V. The epitope map of factor V/Va was determined by using the MAbs (H1, H2, B38, and B10) in a cELISA. Purified factor V, 40 ng in 200 μ L of TBS was coated onto the surface of 96-well polystyrene microtiter plates by incubating overnight at 4°C. The plates subsequently were washed and the remaining sites blocked as described before. The concentrations of MAb-enzyme conjugates to be used were selected based on separate experiments (data not shown). To saturate, all the antigenic sites were added in the absence or presence of 4 to 6X molar excess of unconjugated anti-V IgG. The mixture was incubated overnight and washed as before. Appropriate controls were included as described before to correct for the nonspecific binding. The absorbance resulting from the enzymatic activity of each immunoconjugate bound to the antigen in the absence of any unconjugated antibody was taken to represent the total binding. The absorbance in the presence of other unconjugated MAb was represented relative to the total binding.

Purified human factor Xa and human prothrombin. Factor Xa (125 U/mg) and prothrombin (12 U/mg) were prepared as described by Walsh et al.¹⁵

Effect of factor Xa on MAb binding to factor V/Va. The competition of factor Xa for the binding of the various monoclonal anti-V IgG to the adsorbed factor V/Va in the solid phase system was determined by using ELISA. The polystyrene 96-well plates were coated with factor V/Va as before and incubated with increasing concentrations of factor Xa in 100 μ L of TBS. After two-hour incubation at 4°C, various MAb-enzyme conjugates, predetermined to give the maximal binding to adsorbed factor V/Va, were added in equal volume as factor Xa. The plates were reincubated overnight at 4°C, washed, and the bound anti-V IgG-enzyme conjugates measured as before.

Effect of factor Xa inactivated by (p-Amidinophenyl) methane-sulfonyl fluoride (p-APMSF) on MAb binding to factor V/Va. Factor Xa (2.27 μ mol/L) was inactivated with tenfold molar excess of p-APMSF at room temperature for 30 minutes as described by Laura et al.¹⁶ Factor Xa, treated in the same manner but without the reagent, was used as a control. The enzymatic activity of F-Xa after incubation with p-APMSF measured with the substrate S-2222 was less than 10% that of the control. The effect of inactivated F-Xa on the binding of MAbs was carried out as before.

Effect of prothrombin on MAb binding to factor V/Va. The experiments were carried out as described for the studies on the effect of factor Xa but in the presence of various concentrations of prothrombin.

Effect of Ca^{++} on the monoclonal anti-V IgG binding to factor V/Va. The competition of Ca^{++} for the binding of MAbs was done using ELISA as for factor Xa and prothrombin. Factor V/Va was adsorbed to 96-well microplates and incubated with monoclonal anti-V conjugates in 0.05 mol/L Tris buffer, pH 7.5, containing 4 mmol/L EDTA and 10 mmol/L $MgCl_2$ and increasing concentrations of Ca^{++} . Magnesium chloride was added to the buffer to prevent a pH change owing to the liberation of H^+ ions from EDTA as calcium was added. Using the known equilibrium constants for each of the ionic species in this buffer at this pH, the concentration of the free calcium was calculated by a computer program as described by Perrin and Sayce.¹⁷ Incubation, washing, and detection of the bound antibody-enzyme conjugates were carried out as before.

RESULTS

Human MAbs against human factor V. Previous characterization of MAbs against factor V in a patient revealed that the patient's plasma neutralized factor V coagulant activity in normal plasma to 95% at a ratio of 16:1 (vol/vol)

and that these antibodies were of the immunoglobulin subclasses IgG1 and IgG2 with κ light chain. No factor V antibody activity was associated with subclasses IgG3 and IgG4.⁷ The IgG fraction in the patient's plasma has now been partially purified by precipitation with 45% of saturated ammonium sulfate and applied to a protein A-Sepharose column. Human IgG1 and IgG2 both bound strongly to protein A and were resolved by elution with a pH gradient.⁸ Two peaks of A_{280} , one small peak at about pH 5.2 and another more prominent peak around pH 4.2, were eluted. The latter peak was pooled and characterized as containing IgG1 and κ light chain and used in the studies reported here. SDS-PAGE of the purified anti-V IgG under nonreduced condition showed a single band corresponding to a mol wt of 150,000 daltons and under reduced condition two bands of 50,000 and 25,000 daltons. The IgG1 fraction (0.3 mg/mL) neutralized the factor V coagulant activity in normal plasma almost completely at a ratio of 4:1 (vol/vol).

Epitope Specificity of human MAbs (H2). To determine the antigenic specificity of the antibody,¹²⁵I factor V was cleaved with thrombin and reacted with either antibody (H2) or normal human IgG (control), coupled to Reacti-gel 6X, in the presence of either 20 mmol/L Ca^{++} or EDTA as described in "Methods." The specifically bound component of factor Va was identified as the heavy chain (D polypeptide, mol wt 150,000) by comparison with known factor Va after electrophoresis on a SDS polyacrylamide gel followed by autoradiography (Fig 1). The presence of 20 mmol/L EDTA completely eliminated the binding of factor Va to this immobilized human MAb. Moreover no detectable light chain (F1F2 polypeptide) was adsorbed along with the D polypeptide in the presence of 20 mmol/L Ca^{++} .

Binding specificity of alkaline phosphatase-conjugated and unconjugated MAbs. The activity of the enzyme-conjugate was inversely proportional to the added factor V/Va in a competitive assay. Both conjugated and unconjugated antibody (B10, H1, H2, and B38) gave dose responses that could be described by the same line (data not shown). Thus all the four MAbs, in their abilities to bind to factor V/Va, were unaffected by conjugation.

Dissociation constants (K_D) of purified MAbs for their antigenic determinants. The dissociation constants of antigen-antibody equilibrium in solution was determined using ELISA. When plotted according to the Klotz equation,¹⁴ an excellent fit to a straight line with a correlation coefficient of 0.974 to 0.998 and intercept to ordinate close to 1 were obtained for all the four MAbs. From linear regressions the K_D values obtained were as follows: 1.15×10^{-10} mol/L for B10, 0.30×10^{-10} mol/L for B38, 0.17×10^{-10} mol/L for H1, and 0.78×10^{-10} mol/L for H2.

Epitope mapping of the four MAbs against human factor V. The epitope specifications of the MAbs against human factor V developed and/or purified in our laboratory were examined by reciprocal competitive inhibition of binding of antibody to antigen. Alkaline phosphatase-conjugated MAbs were employed as probes in direct ELISA in the absence or presence of other unconjugated MAbs ("Methods"). As expected, the binding of B10 enzyme-conjugate was inhibited almost completely (100%) in the presence of unconjugated B10 monoclonal IgG (Fig 2). The inhibition was nearly

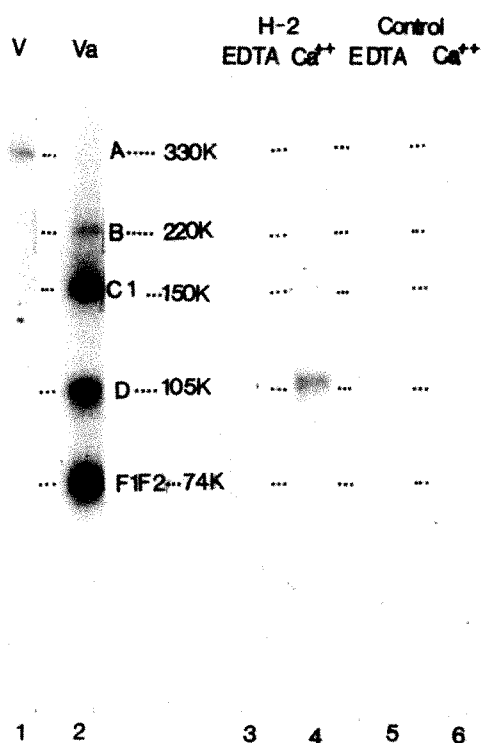


Fig 1. Epitope specificity of the human monoclonal antihuman factor V (H2): autoradiogram of SDS polyacrylamide gel. The affinity-purified monoclonal IgG or normal human IgG, 0.25 mg/mL, was coupled to Reacti-gel 6X in 0.1 mol/L borate buffer pH 8.0. After coupling, the gel-IgG conjugates were centrifuged and resuspended in 0.075 mol/L Tris buffer pH 7.2 containing 0.075 mol/L NaCl, 0.1% BSA, and 20 mmol/L Ca^{++} or 20 mmol/L EDTA. ^{125}I -labeled factor Va, 0.15 μg (0.7×10^6 cpm) was added to each tube and incubated overnight at 4°C with mixing. The gel-ligand mixtures were washed to remove nonspecifically adsorbed proteins. Specifically bound factor Va fragment was dissociated from the complex by boiling in 10% SDS for 10 minutes, electrophoresed on 3% to 15% gradient SDS polyacrylamide gel, and then dried gel applied to autoradiography. Lane 1, factor V; Lane 2, factor Va; Lanes 3 and 4, antibody H2 eluates obtained in the presence of EDTA and Ca^{++} ; Lanes 5 and 6, normal human IgG eluates obtained in the presence of EDTA and Ca^{++} .

80% in the presence of H1, but the presence of B38 or H2 did not interfere with the binding.

The binding of B38 enzyme-conjugate to factor V/Va was totally abolished in the presence of B38. A small degree of inhibition to an extent of 30% and 15% was observed in the presence of B10 and H1 respectively. H2 did not interfere with the binding of B38 enzyme-conjugate to factor V/Va.

With alkaline phosphatase-conjugated H2, the inhibition was total in the presence of unconjugated H2 and strikingly about 72% in the presence of B10. The presence of B38 or H1 did not have any effect on the binding of H2.

The binding of H1 enzyme-conjugate to adsorbed factor V was totally eliminated in the presence of H1 antibody. In the presence of B38, B10, and H2, the binding of H1 anti-V IgG-conjugate was inhibited to about 50%.

Effect of factor Xa on the binding of monoclonal antihuman factor V IgG to factor V/Va. We measured the binding of a predetermined saturating concentration of the

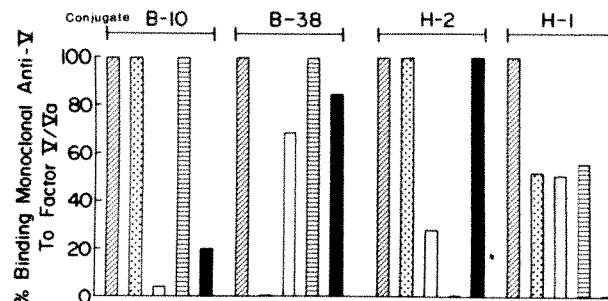


Fig 2. Epitope mapping of the MABs against factor V. Affinity-purified antibodies were conjugated to alkaline phosphatase. Purified factor V/Va, 40 ng in 200 μL of 0.02 mol/L Tris buffer, pH 7.2, containing 0.15 mol/L NaCl was coated onto the surface of 96-well microplates by incubating overnight at 4°C. After incubation the plates were washed with the same buffer containing 1% BSA and reincubated with BSA containing buffer overnight. Monoclonal anti-V IgG-enzyme conjugate in the absence or presence of 4 to 6X excess amounts of various unconjugated MABs were incubated overnight. After washing to remove unadsorbed proteins, the bound antibody-enzyme conjugate was detected by incubating with the substrate of the enzyme. ■, MAB-enzyme conjugate alone; ▨, MAB-enzyme conjugate and unconjugated B38; ▤, MAB-enzyme conjugate and unconjugated B10; ▩, MAB-enzyme conjugate and unconjugated H2; ▪, MAB-enzyme conjugate and unconjugated H1.

various monoclonal anti-V IgG to 40 ng of adsorbed factor Va in the presence of varying amounts of factor Xa ranging from 0.1 $\mu\text{g}/\text{mL}$ to 4.0 $\mu\text{g}/\text{mL}$. As shown in Fig 3, there was a concentration-dependent competition of factor Xa for the binding of the monoclonal anti-V IgG B38, H1, and H2 to factor V/Va. The binding of these antibodies was decreased 80% to 90% in the presence of 4 $\mu\text{g}/\text{mL}$ of factor Xa. In contrast, the binding of B10, an antibody that is neither neutralizing nor directed against factor Va, was reduced only about 20%.

Effect of factor Xa inactivated by (p-Amidinophenyl) methanesulfonyl fluoride (p-APMSF) on MAB binding to factor V/Va. Tracy et al¹⁸ demonstrated that factor Xa proteolytically modified bovine factor Va. Thus we tested whether the decrease in the binding of anti-V IgG B38 to factor V/Va in the presence of added F-Xa was due to proteolysis of factor V/Va by F-Xa. We found that F-Xa incubated with p-APMSF and containing less than 10% of its original activity reduced the binding of B38 virtually identical to that of active F-Xa. Thus at a F-Xa concentration of 0.36 $\mu\text{g}/\text{mL}$, the relative absorbance (A_{405} ; with control without factor Xa defined as 1.0) for active F-Xa was 0.685 of the absorbance in the absence of F-Xa, while for inactivated F-Xa, 0.682. Similarly, at 1.43 $\mu\text{g}/\text{mL}$, the relative absorbance was 0.164 for active F-Xa and 0.160 for inactivated F-Xa. To test whether F-Xa inactivated by p-APMSF could still cleave factor Va, labeled factor Va (0.4×10^{-9} mol/L) was incubated with inactivated F-Xa (2.0×10^{-8} mol/L) overnight at 4°C. The sample was electrophoresed on 3.5% to 15% gradient SDS polyacrylamide gels and subjected to autoradiogram along with a control sample without F-Xa. The autoradiogram of factor Va incubated with inactive F-Xa was identical (data not shown) to that of control (Fig 1). No cleavage of any of the components of

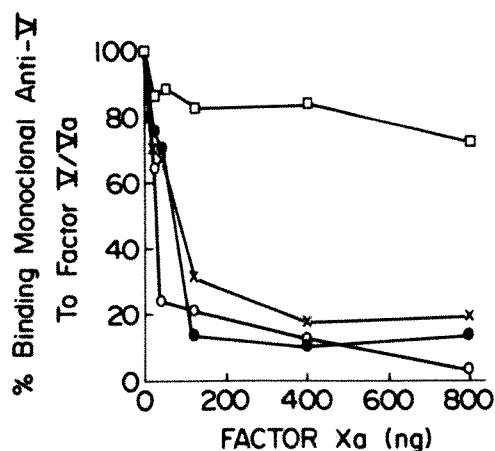


Fig 3. Effect of factor Xa on monoclonal anti-V binding to adsorbed factor V/Va. Microtiter plates were coated with factor V/Va and incubated with antibody-enzyme conjugate along with increasing concentrations of factor Xa (20 ng/200 μ L to 800 ng/200 μ L) overnight. After incubation the plates were washed extensively and the bound antibody-enzyme conjugate was detected by incubating with p-nitrophenyl phosphate 1 mg/mL in 0.05 mol/L sodium carbonate pH 9.8 containing 1 mmol/L $MgCl_2$. $\square$ — $\square$, B10; $\times$ — $\times$, B38; $\bullet$ — $\bullet$, H1; $\circ$ — $\circ$, H2.

factor Va was noted. These results suggested that the effect of F-Xa on the binding of MABs to factor V/Va was not due to proteolytic cleavage of factor V/Va by F-Xa.

Effect of prothrombin on the binding of Antihuman factor V IgG to factor V/Va. The direct binding of thrombin-treated factor V to prothrombin has been demonstrated¹⁹ using partially purified bovine factor V and bovine prothrombin-sepharose affinity column. Therefore we examined the binding of the various MABs to immobilized factor V/Va in the presence of increasing concentrations of human prothrombin ranging from 20 μ g/mL to 375 μ g/mL. Prothrombin at these concentrations did not compete with the binding of any of the MABs to adsorbed factor V/Va, and the binding ranged from 89.4% to 106.3% of the value without added prothrombin.

Effect of Ca^{++} on the binding of Antihuman factor V IgG

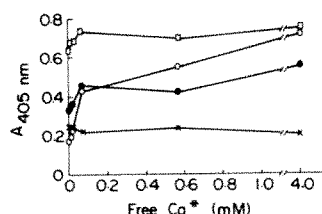


Fig 4. Effect of Ca^{++} on monoclonal anti-V binding to factor V. Factor V/Va was adsorbed to 96-well plates, and various enzyme-conjugated MABs were incubated with increasing amounts of Ca^{++} in 0.05 mol/L Tris buffer, pH 7.5, containing 4 mmol/L EDTA and 10 mmol/L $MgCl_2$. Incubation, washing, and detection of the bound antibody were determined as described in the legend for Fig 3. Free Ca^{++} present in the reaction mixture was calculated by using a computer program described in "Methods." In the absence of added Ca^{++} , the reaction mixture does not contain any free Ca^{++} . $\square$ — $\square$, B10; $\times$ — $\times$, B38; $\bullet$ — $\bullet$, H1; $\circ$ — $\circ$, H2.

to factor V/Va. Metal ions such as Ca^{++} , Mn^{++} , Co^{++} , and Cd^{++} have been shown to maintain bovine factor Va activity.²⁰ An association and/or direct binding of calcium to factor V/Va has also been observed.^{21,23} We examined the effect of Ca^{++} on the binding of the different MABs that have distinct epitope specificities to factor V/Va as described in "Methods." As shown in Fig 4, all four MABs had the ability to bind to adsorbed factor V/Va in the absence of added Ca^{++} . However, in the presence of varying concentrations of Ca^{++} in the incubation mixture, their ability to bind differed strikingly. An increase in the binding of the two human monoclonal antibodies, H1 and H2, was noted as the physiologic concentration of Ca^{++} (1.3 mmol/L) was approached. At 4 mmol/L, the absorbance related to the binding of H1 increased 1.65-fold, while that of H2 increased 3.65-fold. In contrast, there was little or no effect on the binding of the two murine MABs.

DISCUSSION

Previously we described two MABs to factor V in a patient that were typed as a mixture of IgG₁ of IgG₂ with κ light chains. We have now purified an IgG₁ fraction containing the MAB (H2) from the plasma of this patient and determined that it is directed against the heavy chain of factor Va (D polypeptide). This fraction contains an antibody that contains a single class of heavy chain and light chain, a single class of binding sites with a K_D of 0.78×10^{-10} mol/L, and reacts with the single chain of factor V. These data strongly suggest that it is a "monoclonal" antibody, although absolute proof for a naturally occurring human antibody is difficult to obtain. This report is the first description of a MAB to human factor V/Va, which recognizes an antigenic site on the D chain of human factor Va.

The requirement of two polypeptides bridged by Ca^{++} for the integrity of factor Va activity is well established.^{21,24} Using anti-bovine factor V MABs, Tracy and Mann²⁵ and Tucker et al²⁶ have described the functional significance of light chain of factor Va in some detail and found that it is involved in the interaction with factor Xa and platelets. The phospholipid or platelet-bound factor Va is inactivated by activated protein C as a result of the cleavage of D polypeptide, and factor Xa protects against this inactivation,^{18,27,28} suggesting the possibility that factor Xa also interacts with the heavy chain of factor Va. However, the exact contribution of D polypeptide of human factor Va to the function of prothrombinase complex is not established. By using this human MAB (H2) as probe, we have defined more specifically the role of D polypeptide of human factor Va.

In determining the epitope specificity of the human MAB (H2) we have made two observations that are unexpectedly different from results with antibodies to the D chain of bovine factor V and hence interesting. First, in the presence of 20 mmol/L EDTA the labeled factor Va was not immunoprecipitated with the antibody H2, in contrast to its behavior in the presence of 20 mmol/L Ca^{++} . It may be that the binding of this antibody to factor V/Va is weak in the presence of EDTA and the antigen lost during extensive washing with the buffer containing detergent and high salt.

Second, only the heavy chain (D) of factor Va binds to H2 in the presence of Ca^{++} , and the light chain (F1F2) is not coadsorbed. This observation indicates that the binding of MAb may disrupt the Ca^{++} -mediated association of the two components of factor Va. This effect could arise through steric hinderance, conformational changes, or binding at or near the Ca^{++} binding site in the D polypeptide. These results contrast with the observations of Foster et al with the bovine factor V.³ The two monoclonal bovine anti-factor V antibodies, $\alpha_1\text{D}$, $\alpha_2\text{D}$ (D polypeptide-specific) and $\alpha_2\text{E}$ (the light chain or E polypeptide-specific) immunoprecipitate both the heavy and light chain of bovine factor Va in the presence of Ca^{++} . With bovine factor V, therefore, the binding of antibody to the D or E polypeptide does not perturb the integrity of the two-subunit bovine factor Va molecule in the presence of Ca^{++} . Wilson et al⁵ has determined that the human MAb (H1), reported by Coots et al,⁴ is light-chain specific. They find that the heavy chain is immunoprecipitated to a much lesser extent compared to the light chain of labeled factor Va with the antibody in the presence of Ca^{++} . This finding is similar to what we observed with the human MAb (H2). We have also found this preferential behavior with the murine monoclonal antihuman factor V, B38 (Doshi and Colman, unpublished observation). Thus this result reflects a weaker Ca^{++} dependent association between the two chains of factor Va in human compared to that in bovine plasma. These results are also consistent with the observations that bovine factor Va is more stable than the human factor Va, which in part may be due to tighter calcium binding.

The dissociation constants obtained for the four antibodies varied from 0.17 to 1.15×10^{-10} mol/L, indicating that the Mabs are of high affinity. The two human antibodies (H1 and H2) each may represent only a portion of the purified IgG preparation, which also probably contains multiple antibodies to different antigens. The method employed in the studies here allows the measurement of true K_D in solution of antibody-antigen complexes by ELISA, since the derived equation is independent of the absolute antibody concentration and depends on the relative amount of free antibody and the total antigen concentration. By separating the IgG1 from IgG2 in H2, we have avoided the only difficulty if these two antibodies to factor V should have overlapping specificity. Since IgG4 elutes in small amounts in both the IgG1 and IgG2 fraction, the possibility that IgG4 is the responsible species must be considered. However, Chiu et al⁷ have shown previously that only IgG1 and IgG2 subclasses contain antibodies that bind to factor V in an ELISA assay.

The data obtained from the reciprocal competitive inhibition of the binding of antibodies to the antigen provide insight into the spatial relationship of the polypeptide domains in the human factor V/Va molecule. The electron microscopic structure of human factor V/Va of Dahlback et al²⁹ provides a framework for interpretation (Fig 5), although the identification of each polypeptide is not certain. In all cases the unconjugated antibody completely competes with the same conjugated antibody, thereby further validating the system. The uninhibited binding of antibody-conjugate to the antigen in the presence of excess amounts of

other unconjugated antibodies is taken to indicate that the two epitopes are independent. The scheme is conceptually useful but does not imply any firm conclusion about actual molecular distances.

Partial inhibition observed between some antibodies could be explained by weak or strong steric hinderance between the conjugated and inhibitor antibodies, inducing an apparent decrease in the binding of the antibody-enzyme conjugate to the antigen. Human MAb H1 and mouse MAb B38 are both specific to the F1F2 polypeptide of human factor Va, and B10 mouse MAb is specific to the activation peptide. The binding of B10 conjugate, as shown in Fig 2, is reduced by 80% in the presence of H1 but remains unaffected in the presence of B38. The data indicate first that the epitopes of B38 and H1 in the light chain of factor Va are distinct and second that the antigenic determinant of H1 may be in close proximity to the epitope of B10 antibody in the activation peptide.

Binding, kinetic, and immunochemical evidence all support a close interaction between factor Va and factor Xa.³⁰ Our results provide evidence that factor Xa can interact with factor Va in the absence of platelets or phospholipids. The failure of factor Xa to inhibit the binding of B10 suggests that factor Xa interacts with factor Va. Tucker et al²⁶ have found that the interaction of bovine factor Va and factor Xa in the presence of phospholipids is inhibited by an antibovine factor V MAb directed against the light chain. Tracy and Mann²⁵ have suggested that the light chain of bovine factor Va bound to platelets also serves as the binding site for factor Xa. Since the MAb B38 and H1 used in this study are both directed against the light chain of human factor Va,^{5,6} total inhibition of the binding of these two antibodies to factor Va by factor Xa is consistent with the earlier findings.

Previous studies present kinetic evidence for the interaction of polypeptide D of factor Va and factor Xa.^{18,27,28} Factor Va is inactivated by activated protein C by cleavage of polypeptide D, and factor Xa protects factor Va from this inactivation. Our observation in this paper is that the binding of H2 MAb, which is specific for the D polypeptide of factor Va (Fig 1), is inhibited competitively in the presence of increasing concentrations of factor Xa, which demonstrates existence of such an interaction between D polypeptide of factor Va and factor Xa. This finding is consistent with the previous observation that cleavage of the D polypeptide of bovine factor Va by protein C destroys its ability to bind to immobilized inactivated factor Xa.³¹ The possibility that our results are due to factor Xa cleavage of factor Va is ruled out by the similar behavior of inactive factor Xa.

Esmon et al¹⁹ have demonstrated the direct binding of bovine factor Va to prothrombin. More recently, Guinto and Esmon³¹ have demonstrated that the bovine factor Va heavy chain binds to prothrombin in a specific and Ca^{++} independent fashion. However, we find that prothrombin, even at concentrations higher than that of plasma, 375 $\mu\text{g}/\text{mL}$ does not inhibit the binding of any of the MAb tested. The prothrombin binding site in factor Va may be distinct from the epitopes recognized by these MAb. Alternatively, prothrombin may bind only to phospholipid or platelets or to a factor Va-lipid complex. Prothrombin may require factor Xa

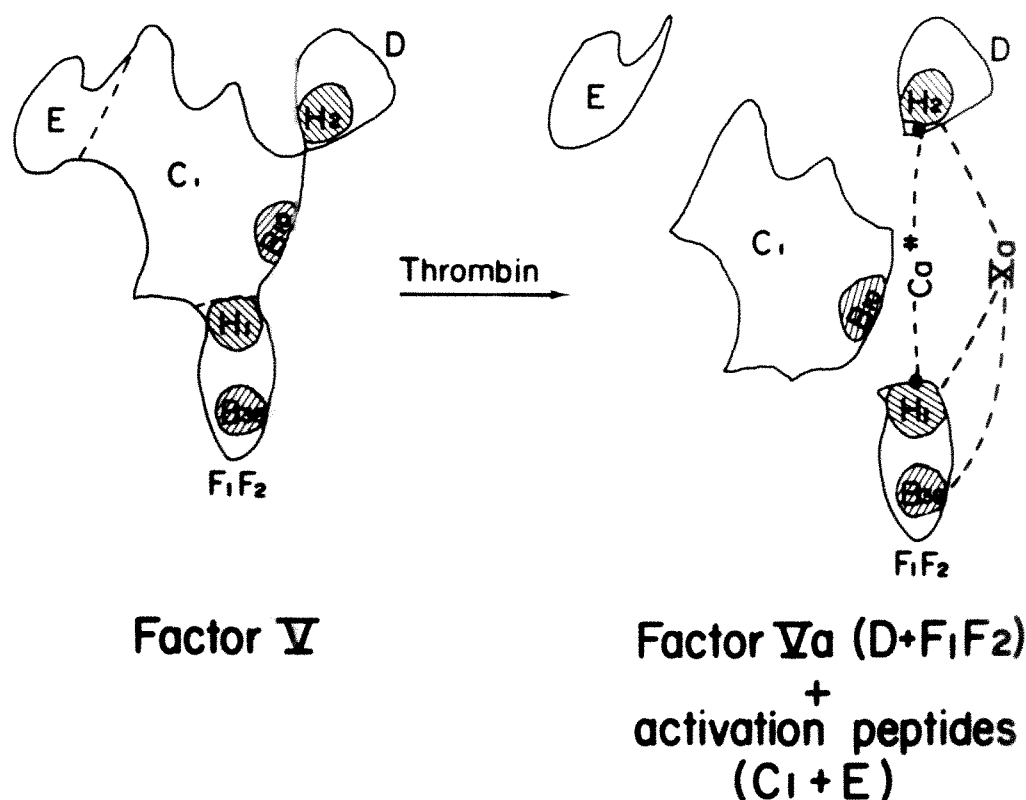


Fig 5. Schematic diagram of factor V/Va functional epitopes. When the single chain factor V is activated to factor Va by thrombin, a number of polypeptides are formed. They are D (mol wt 105,000); F₁F₂ (mol wt 71,000 to 74,000); C₁ (mol wt 150,000), and E (mol wt 71,000). Functional domains and topographic relationships are defined from the data obtained in competitive inhibition experiments using human factor V MAbs, which recognize distinct epitopes in factor V/Va molecule.

to promote binding or may not bind to factor V/Va immobilized on plastic. Rosing et al³² have shown that phosphatidylserine exposed at the outer surface of stimulated platelets by collagen and thrombin promotes prothrombin and factor X activation. However, these kinetic studies do not distinguish between the above alternatives.

The effect of Ca⁺⁺ on the binding of these MAbs supports the results from competitive ELISA that the murine MAb B38 and human MAb H1 recognize distinct epitopes in the F₁F₂ polypeptide of factor Va. The binding of both human MAb (H1 and H2) to the antigen is increased by Ca⁺⁺, although the effect is more pronounced for H2 in contrast to the murine MAbs (B38 and B10). Hibbard and Mann have demonstrated three calcium-binding sites.²² It is likely that in

the presence of 4 mmol/L-free Ca⁺⁺, which is several fold excess of the reported values of the dissociation constants for the high and low affinity binding sites of Ca⁺⁺, all the sites are occupied. There may be an interaction between one or more of these Ca⁺⁺ sites and the epitopes recognized by the human MAbs. The data, however, do not distinguish between an increased number of antibody binding sites or an increased affinity of the antibody binding to factor V/Va.

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A New Mutation in IVS-1 of the Human β Globin Gene Causing β Thalassemia Due to Abnormal Splicing

By George F. Atweh, Corinne Wong, Robin Reed, Stylianos E. Antonarakis, Ding-er Zhu, Prahbat K. Ghosh, Tom Maniatis, Bernard G. Forget, and Haig H. Kazazian, Jr

A G to T transversion at the fifth nucleotide of the first intervening sequence (IVS-1) of the β -globin gene has been identified in cloned β -thalassemia genes of two unrelated individuals, one of Mediterranean and the other of Anglo Saxon ancestry. In each patient the mutation was present in a different β globin gene framework, defined by intragenic restriction site polymorphisms, thereby suggesting the occurrence of independent mutations. The study of the RNA products of one of these cloned genes, after transfer

and transient expression in HeLa cells, showed partial inactivation of the normal donor splice site of IVS-1 and activation of two major and one minor cryptic splice sites. Only one of the two major cryptic sites was utilized in a cell-free splicing extract. The effects of this mutation on messenger RNA (mRNA) splicing are similar to that of another β thalassemia gene with a G to C transition at the same position.

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β -THALASSEMIA is one of the commonest single gene disorders that has been recognized and studied by geneticists and hematologists for several decades.^{1,2} The interest in this disorder has recently intensified with the advent of recombinant DNA technology. Mediterranean and other populations have been investigated in great detail at the molecular level, and numerous lessons about gene structure and function in health and disease have been learned from these studies.^{3,4} The systematic approach for the study of this disease, advocated by Orkin et al.,⁴ has allowed the identification of a large number of mutant β -thalassemia genes. This approach is based on the strong correlation between specific mutations and certain chromosomal backgrounds, called haplotypes, defined by patterns of restriction fragment-length polymorphisms (RFLPs) in the β -globin gene cluster. In general, a given mutation is associated with a particular haplotype in a certain racial group. A survey of 164 Mediterranean β -thalassemic alleles has revealed a correlation of 86% between a given haplotype and a given thalassemic mutation.⁵ Using this approach we have characterized a new mutation from two unrelated individuals of different ethnic origins. This mutation changes the fifth nucleotide of the first intervening sequence (IVS-1 position 5) from G to T and alters the pattern of RNA processing.

MATERIALS AND METHODS

High mol-wt DNA was isolated from the spleen or leukocytes⁶ of affected individuals using the method of Blin and Stafford.⁷ Haplotype determination was performed by restriction endonuclease analysis⁸ using the same combination of restriction enzymes and DNA probes as that described by Orkin et al.⁴ The β -globin genes were cloned as 7.5 kb *Hind* III fragments, as described earlier.^{6,9} The Mediterranean β -thalassemia gene was subcloned as a 3.7 kb *Pst* I/*Bgl* II fragment in the expression vector π SVplac.¹⁰ DNA sequence analysis was performed by the dideoxy chain termination method of Sanger et al.¹¹

π SVplac recombinants containing a normal β -globin gene¹⁰ or a β -thalassemia gene were used to transfect HeLa cells at 25% confluency using the technique of DNA coprecipitation with calcium phosphate followed by glycerol shock, as described earlier.⁶ After 36 hours the cells were harvested, and cytoplasmic RNA was extracted using methods described by Favaloro et al.¹² The extracted RNA was used in S1 nuclease mapping experiments¹³ with either uniformly labeled single-stranded probes or 3' end-labeled double-stranded probes (see "Results" for description of specific probes). After S1 nuclease digestion, the protected DNA fragments were fractionated by electrophoresis in 5% or 8% polyacrylamide gels in

the presence of 7 mol/L urea. The products of RNA processing were also evaluated by primer extension studies.¹⁴ The RNA isolated from transfected HeLa cells was annealed to a 30-base synthetic oligonucleotide primer complementary to the RNA strand of exon 2, and cDNA copies of the RNA were synthesized using viral reverse transcriptase, as described.¹⁴ The products were analyzed by electrophoresis in an 8% urea-polyacrylamide gel using appropriate size markers. Cell-free splicing of truncated precursor β globin mRNA molecules, synthesized in vitro by use of the SP6 promoter/vector system,¹⁵ was carried out as previously described.^{16,17}

RESULTS

Cloning and sequence analysis of β -thalassemic genes. Haplotype analysis of the Mediterranean patient revealed heterozygosity for haplotypes Va and Vb, which differ only at the polymorphic *Hinf* I site 5' to the β gene⁵ (Fig 1). One of the two β -thalassemia genes from this patient was studied earlier by Treisman et al.¹⁰ and was found to contain a G to A transversion at IVS 2 position 1. This mutation abolishes the *Hph* I restriction endonuclease site normally present at the exon 2/IVS-2 junction and is commonly associated with haplotype Vb.⁵ Haplotype Va, on the other hand, is commonly associated with a G to T substitution at IVS-1 position 1. Hybridization studies using synthetic oligonucleotides ruled out the presence of this mutation in the patient's DNA (data not shown). The β -thalassemia gene from the haplotype Va chromosome was cloned and identified by the retention of the *Hph* I recognition site normally present at the IVS-2 donor splice site. Sequence

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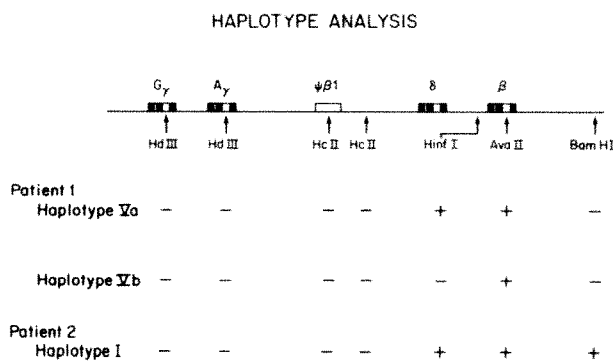


Fig 1. Results of haplotype analysis in the two patients. Haplotype Va is commonly associated with a G → T substitution at IVS1 position 1; haplotype Vb is commonly associated with a G A substitution at IVS-2 position 1,⁸ and haplotype I is often associated with either a G → A transition at IVS-1 position 110 or β-nonsense 39.

analysis of the promoter region, the three exons, and all intron-exon junctions of this gene revealed, as the only abnormality, a G to T transversion at IVS 1 position 5 (Fig 2).

Haplotype analysis was also performed on a nuclear family of Northern European ancestry. The thalassemia allele was of haplotype I (Fig 1) and was inherited from both parents, who were first cousins, facilitating cloning of the β-globin gene from the homozygous patient. The two most frequent mutations associated with this commonest Mediterranean chromosome background are the G to A transition at IVS-1 position 110 and the β-39 nonsense mutation.⁵ Both mutations were shown, by oligonucleotide hybridization, to be absent in this patient (data not shown), thereby making this gene a candidate for a new mutation. Nucleotide

sequence analysis using synthetic oligonucleotide primers revealed a G to T substitution at IVS-1 nucleotide 5, the same mutation identified in the first patient described above.

Gene transfer and gene expression studies in HeLa cells. Processing of RNA transcribed from the Mediterranean β-thalassemic gene was studied by transfecting in parallel a normal β-globin gene and the thalassemic gene, both cloned in the expression vector πSVplac, into cultured HeLa cells. The cytoplasmic RNA was isolated and subjected to S1 mapping studies using a uniformly labeled single-stranded probe that extends from the promoter of the β-globin gene to the Eco RI site in exon 3¹⁸ (Fig 3A). RNA transcribed from the normal β-globin gene protects a 223-nt fragment corresponding to exon 2 and a 143-nt fragment corresponding to exon 1. The 49-nt fragment corresponding to exon 3 can only be seen on high percentage gels (data not shown). RNA transcribed from the β-thalassemic gene with the G to T mutation at IVS-1 position 5 (IVS1-5^T) protects normal amounts of the 223-nt fragment corresponding to exon 2, decreased amounts of the 143-nt fragment corresponding to exon 1, and two additional fragments, 128 and 105 nt in size. Transfection of HeLa cells with a different cloned β-thalassemic gene containing a G to C mutation at IVS-1 position 5¹⁹ gave a qualitatively similar pattern of S1 nuclease protection (data not shown). A similar pattern was also obtained using reticulocyte RNA from the Mediterranean patient (data not shown), thus demonstrating that the same splicing defects occur in vivo; an additional band of 270 nt was also detected corresponding to the alternatively spliced β-globin mRNA from the patient's other β-thalassemic gene carrying a point mutation at IVS-2 position 1.¹⁰

Splicing at the IVS-1 donor splice site was also evaluated using a 3' end-labeled *Hgi* AI fragment (Fig 3B) as a probe

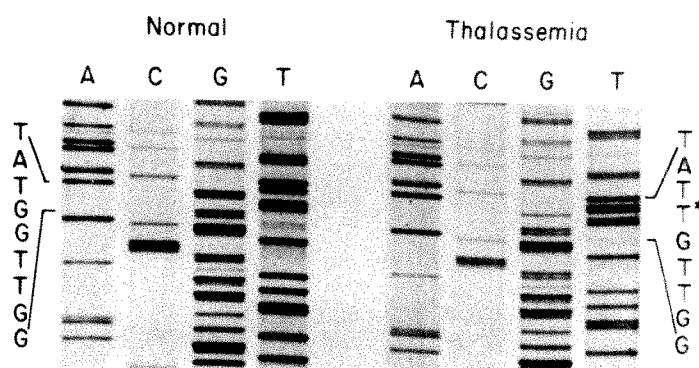
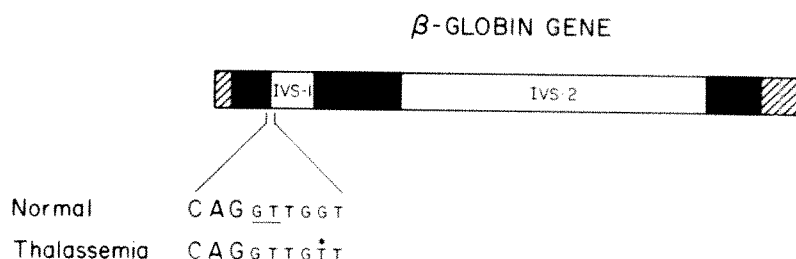


Fig 2. Side-by-side comparison of the DNA sequence of the Mediterranean β-thalassemic gene from haplotype Va and a normal β-globin gene in the region of the IVS-1 donor splice site. The mutated nucleotide (G) is denoted by (*). The schematic diagram of the β-globin gene shows the position of this mutation at the fifth position of IVS-1. The GT invariant dinucleotides of the donor splice site are underlined.



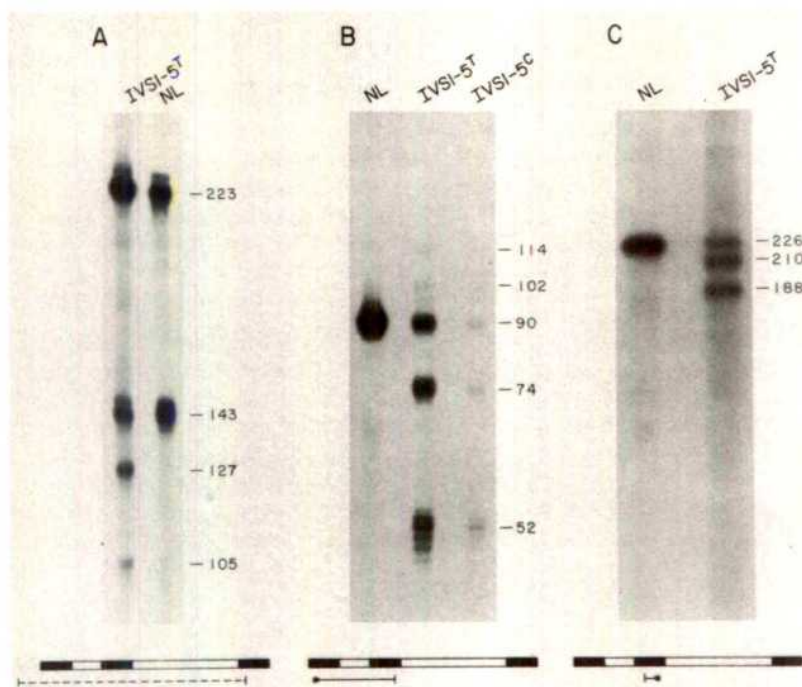


Fig 3. Analysis of RNA resulting from transient expression of β -globin genes after transfer into HeLa cells. (A) S1 nuclease mapping study of RNA transcribed from the IVS1-5^T β -thalassemic gene and a normal β -globin gene using a uniformly labeled single-stranded probe represented on the schematic map below the autoradiograph. The 223-nt fragment (exon 2) and the 143-nt fragment (exon 1) are seen in the normal (NL) lane. Additional fragments (128 nt and 105 nt) are seen in the IVS1-5^T lane. (B) S1 nuclease mapping of the same RNAs and RNA from the IVS1-5^C gene using a 3' end-labeled probe represented on the schematic map below the autoradiograph. A 90-nt fragment is seen in the NL lane that corresponds to the part of exon 1 complementary to the probe. Both IVS1-5^T and IVS1-5^C lanes show decreased amounts of the 90-nt fragment in addition to three other fragments (102, 74, and 52 nt); a fourth 114-nt fragment of unknown significance is present in all lanes. (C) cDNA products of a primer extension study using RNA from the IVS1-5^T gene or a normal gene (NL) and a primer from exon 2 represented on the map below the autoradiograph. The NL lane shows the expected 226-nt cDNA product, while the IVS1-5^T lane shows decreased amounts of the 226-nt product in addition to two abnormal products (210 and 188 nt).

in another S1 nuclease mapping experiment. The autoradiograph in Fig 3B shows the expected 90-nt fragment when RNA from the normal gene was used and four additional fragments when RNA from the IVS1-5^T gene was used. The 52- and 74-nt fragments correspond to the aberrantly spliced RNAs that generated the fragments of 105 and 127 nt respectively in Fig 3A. These protected fragments result from the utilization of cryptic splice sites at codons 18 and 25 in exon 1.¹⁹ The 102-nt fragment is consistent with the utilization of a cryptic splice site at position 12 of IVS-1.¹⁹ This donor splice site is utilized much less efficiently than the cryptic splice sites in exon 1. The fragment of 114 nt was seen with the normal β gene as well as the β -thalassemic gene. The significance of this fragment is not clear; however, its size is consistent with a splicing event at a cryptic donor splice site at position 24 of IVS-2. A qualitatively similar pattern of splicing was obtained with the β -thalassemic gene containing a G to C mutation at IVS-1 position 5 (IVS1-5^C) (Fig 3B, lane 3). The difference in intensity of the bands between RNA from IVS1-5^T (lane 2) and that of IVS1-5^C (lane 3) is probably due to differences in transfection efficiency of the two genes in the particular experiment shown.

RNA processing was further evaluated by a primer extension study using a 5' end-labeled synthetic oligonucleotide, complementary to the mRNA sense-strand of exon 2, as a

primer in the reverse transcription reaction. The cDNA product of the RNA transcribed from the normal gene is 226 nt in size (Fig 3C). RNA from the IVS1-5^T gene directed the synthesis of cDNA products of 226, 210, and 188 nt in size. The abnormal cDNA products (210 and 188 nt) have sizes consistent with mRNA abnormally spliced at the two cryptic splice sites within exon 1. The minor species thought to result from splicing at the cryptic splice sites within IVS-1 are not visualized in this study.

In vitro splicing in the cell-free extract. To determine the effects of the IVS1-5^T mutation on the in vitro splicing of human β -globin pre-mRNA, we examined both normal and thalassemic SP6 precursors containing exon 1, IVS-1, and exon 2 in the HeLa cell nuclear extract.^{16,17} The results of this analysis are shown in Fig 4. Splicing products and intermediates were identified by comparison with the known species generated from the normal precursor.^{16,17} In addition, splicing intermediates were identified by examining the products generated when the in vitro reaction was carried out in the absence of KCl. Under these conditions only products from the first step of the reaction, exon 1 and the IVS-1/exon 2 lariat, are observed.¹⁶ These analyses revealed that the predominant splicing product generated from the IVS1-5^T precursor is derived from utilization of the cryptic 5' splice site in codon 25 of exon 1. In addition, some normally spliced RNA is observed. However, by contrast with the in vivo

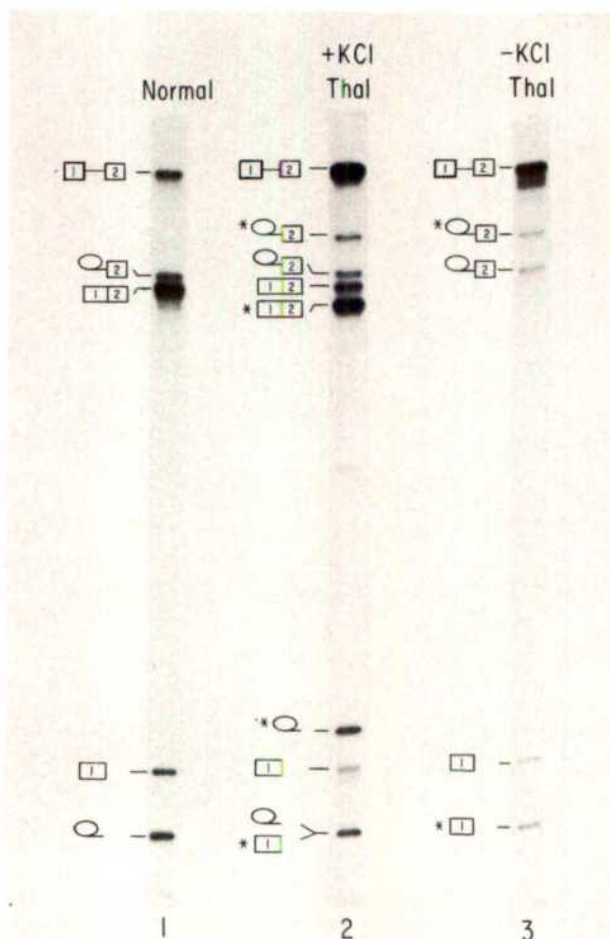


Fig 4. In vitro splicing of normal and IVS1-5^T human β -globin precursors. ³²P-labeled SP6 precursors were synthesized in vitro,¹⁷ spliced in the HeLa cell nuclear extract,¹⁸ and then fractionated on a 5% denaturing polyacrylamide gel. The structures of each of the RNA species are indicated adjacent to the autoradiogram. The boxes refer to exons, and the line indicates the intron. The closed loop indicates the lariat form of the intron. The mutant products and intermediates are designated by an asterisk. The unlabeled faint band seen in lane 2 (also barely visible in lane 1) results from exonuclease degradation of the small amount of uncapped mRNA in the reaction mixture.^{16,17} The sizes of the bands are as follows: unspliced precursor: 497 nt; exon 1/exon 2: 367 nt; and exon 1: 155 nt; the corresponding mutant products are shorter by 16 nt. Lane 1: Normal precursor; Lane 2: Precursor carrying IVS1-5^T mutation, KC1 in the splicing reaction; Lane 3: Precursor carrying IVS1-5^T mutation, KC1 absent from the splicing reaction.

results, no evidence was obtained for utilization in vitro of the cryptic 5' splice site at codon 18. Figure 4 also shows that the splicing efficiency of the thalassemic precursor is less than that of the normal precursor, probably as a consequence of competitive interactions between the normal and mutant 5' splice sites.

To determine whether the normal site of lariat formation, at an adenine residue 37 nucleotides upstream from the 3' splice site, is also utilized when the cryptic 5' splice site is activated, we localized the branchpoint in the mutant IVS-1.

Using the previously developed branchpoint mapping technique,^{17,20} we found that the site of lariat formation occurs at the same position in both the mutant and normal introns (data not shown).

DISCUSSION

The Mediterranean patient studied here has severe transfusion-dependent β -thalassemia major resulting from a profound decrease in β -chain synthesis. One of the two β -globin genes from this patient was studied earlier by Treisman et al¹⁰ and was found to have a mutation that converts the GT invariant dinucleotide of the IVS-2 donor splice site to an AT and thus abolishes normal splicing completely at that site. Haplotype analysis of polymorphic restriction sites in the β -globin gene cluster in this patient revealed heterozygosity for haplotypes Va and Vb. Haplotype Vb is the one commonly associated with the above GT to AT base substitution.⁵ Haplotype Va, on the other hand, is commonly associated with a G to T base substitution at IVS-1 position 1.⁵ Hybridization studies using synthetic oligonucleotides ruled out the presence of the latter mutation in this patient's DNA. DNA sequence analysis of the haplotype Va gene in our patient revealed a G to T transversion at IVS 1 position 5. This mutation is within the consensus sequence of the donor splice site,²¹ a region that is believed to be critical for accurate mRNA splicing.

The second patient in this study differs from the first patient in terms of geographic origins (Northern v Southern Europe) as well as in haplotypes (I v Va; Fig 1) and β -globin gene frameworks (1 v 2).⁴ This patient also differs in that he is homozygous for the mutation, as his parents are first cousins. His clinical phenotype is also typical of transfusion-dependent β -thalassemia major. Despite the difference in chromosome backgrounds, however, both patients have the same mutation of RNA splicing. Both cases represent instances in which the expected mutation/haplotype correspondence was absent. As shown here and emphasized in another survey of seven thalassemia genes,²² this close association of a specific mutation and a particular haplotype is not invariant. Several examples of mutation spread to different ethnic groups, and disparate chromosome backgrounds have been observed and have provided strong evidence for independent origins of the same mutation.^{3,22}

For the β^+ mutation to migrate from haplotype I to Va and framework 1 to 2, a crossing-over event between the mutation and IVS-2 position 74 is required. Occurrence of a recombination event within a short stretch of DNA is less likely than two independent origins of the same mutation in the two ethnic groups. It is of interest that of a relatively small number of β -thalassemia mutations (7 of 37 known mutations) in which recurrences have occurred, two are mutations at the same nucleotide, IVS-1 nt 5.

When we introduced the cloned Mediterranean thalassemic gene into HeLa cells using a transient expression vector and analyzed its mRNA products in S1 nuclease mapping experiments, we demonstrated decreased utilization of the usual IVS-1 donor splice site and utilization of at least three surrounding cryptic splice sites. Two of these cryptic splice sites are in exon 1 of the gene and one is in

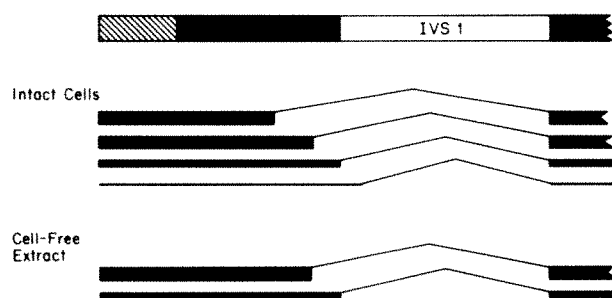


Fig 5. Splicing patterns of the β -thalassemic pre-mRNA in intact cells (A) and in the cell-free extract (B). The approximate relative amounts of the different spliced mRNAs are indicated by the thickness of the individual lines.

IVS-1. As illustrated in Fig 5, the majority of the RNA products that accumulated in HeLa cells were spliced at the two cryptic splice sites within exon 1. These cryptic splice sites are also commonly utilized in other β -thalassemic globin genes that carry different mutations within the con-

sensus sequence of the IVS-1 5' splice site,¹⁹ including a β -thalassemia gene isolated earlier from an Asian Indian patient that was shown to have a G to C transition at IVS-1 position 5,²³ the same position that is mutated in the genes from our patients. Splicing of the mutant precursor mRNA in a cell-free extract also revealed decreased utilization of the normal splice site but, in contrast to the results in intact cells, only one of the cryptic sites in exon 1 was utilized (Fig 5). Similar differences in the relative amounts of spliced RNAs obtained from cryptic sites have been observed with most of the other thalassemia mutants that have been analyzed both in vivo and in vitro.^{16,17,20,23} This may be due to differences in the concentrations of the various factors in intact cells compared to that in the in vitro extracts.²⁴

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Endothelial Cell Interactions With Sickle Cell, Sickie Trait, Mechanically Injured, and Normal Erythrocytes Under Controlled Flow

By Gilda A. Barabino, Larry V. McIntire, Suzanne G. Eskin, David A. Sears, and Mark Udden

Increased adhesive forces between sickle erythrocytes and endothelial cells (EC) have been hypothesized to play a role in the initiation of vasoocclusion in sickle cell anemia. Erythrocyte/human umbilical vein EC interactions were studied under controlled flow conditions for normal (AA), homozygous sickle cell (SS), sickle cell trait (AS), mechanically injured normal, and "high-reticulocyte control" RBC by using video microscopy and digital image processing. The number of adherent RBC was determined at ten-minute intervals during a washout period. Results indicate that SS RBC were more adherent than AA RBC. Mechanically injured (sheared) AA RBC were also more adherent than control normal cells but less adherent than SS RBC. AS RBC did not differ significantly in their adhesive properties from normal RBC. Less-dense RBC were more adher-

ent to EC than dense cells for normal, SS, and high-reticulocyte control RBC. The number of cells adherent at a given time during washout was a very strong function of wall shear rate. In addition, at all shear rates studied, the average velocity of individual SS RBC in the region near the EC surface was approximately half that of AA RBC at the same bulk volumetric flow rate through the flow chamber. These findings suggest that the increased adhesion of sickle RBC is at least partially related to the increased numbers of less-dense RBC present. Increased adherence of the less-dense cells to the EC lining vessel walls could contribute to microvascular occlusion by lengthening vascular transit times of other sickle cells.

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RBC-ENDOTHELIAL CELL (EC) interactions may be important in the vasoocclusive phenomena observed in sickle cell disease. Evidence has been presented by several investigators that suggests that sickle cells adhere abnormally to EC under static conditions as well as under uncontrolled conditions of flow.¹⁻⁶ The abnormal adherence of sickle cells has been correlated with the vasoocclusive severity of the disease.⁷ Interactions between blood cells and endothelial surfaces in blood vessels occur under dynamic conditions. Flow inhibition or promotion of blood cell adhesion to the endothelium may occur depending on the velocity and morphology of the moving cells. Presumably, cells in the vicinity of the endothelium that are moving slowly would have a greater chance of making contact and interacting with the endothelial surface.

Altered flow characteristics of sickle blood under both oxygenated and deoxygenated conditions have been reported in microvascular studies using intravital microscopy techniques.^{8,9} LaCelle¹⁰ reported that adherence of sickle cells to endothelium occurred at low flow rates. Klug and Lessin⁸ reported that sickle RBC adherence to the vessel wall did not occur at moderate and high flow rates. The mean transit time of sickle RBC was higher than that for normal RBC during their passage through the coronary circulation. In a rat mesocecum microvascular preparation,¹¹ it appeared that the cells actually trapped are mainly the dense sickle RBC.

The shear rate (flow rate) is an important dynamic variable present in vivo that will affect the propensity of

erythrocytes to adhere to the vessel wall. In the studies described later, we consider the case of laminar flow between two parallel surfaces as a simplified model of a blood vessel. In this case, movement of fluid in the direction of the applied pressure gradient results in the development of a velocity gradient within the fluid. The velocity gradient, or shear rate, is defined as the difference in velocity between two adjacent layers of moving fluid divided by the distance between them and has the units (cm/s)/cm, or "inverse seconds" (s^{-1}). Time average wall shear rates vary throughout the circulation and range from 0 to 5,000 s^{-1} . The highest values occur in the arterioles and microcirculatory vessels and the lowest values in the small venules and veins. There is a rapid drop in the average vessel wall shear rate as a cell exits the capillaries and enters the venules. Using a jet shearing technique, Smith and LaCelle¹² have recently shown that the number of sickle cells adherent to cultured endothelium decreased as the wall shear rate produced by the fluid flow was increased after a 30-minute static incubation.

In our studies, the effect of wall shear rate on the adhesion of RBC to EC was examined under controlled flow conditions. Velocity gradients utilized in these experiments approximate those found in venules, which are probably important sites for adhesive events. Leukocyte margination in vivo is usually seen to occur in this part of the circulation. Fluid drag forces on attached cells (related to the wall shear rate) are quite low in these vessels. Videotaped experiments were analyzed by using digital image analysis techniques to determine the velocities of individual sickle and normal RBC in the vicinity of a cultured human endothelial cell monolayer.

MATERIALS AND METHODS

Blood was drawn into heparinized tubes from patients with homozygous sickle cell disease (SS), volunteers with sickle cell trait (AS), other patients with high reticulocyte counts ("high-reticulocyte controls"), and normal subjects (AA). After centrifugation at 1,000 g for ten minutes, the plasma and buffy coat were removed. The RBC were washed three times with medium 199 supplemented with 20% fetal calf serum (FCS, Hyclone Tissue Culture Products, Logan, UT), 100 U/mL penicillin, and 100 μ g/mL streptomycin and then suspended at the desired hematocrit in the supplemented medium. For experiments involving density-fractionated RBC, cells

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in plasma were separated into density fractions by centrifugation at 30,000 g for one hour at 30°C in an angle rotor.¹³ The column of RBC was separated into five equal fractions. Sheared RBC were obtained by subjecting suspensions of cells to a subhemolytic shear stress of 1,500 dyne/cm² for two minutes in a Rice University concentric cylinder viscometer described previously.¹⁴⁻¹⁶

Endothelial cell cultures. Human umbilical vein EC were harvested from umbilical cords by using culture procedures adapted from those of Gimbrone et al¹⁷ and grown to confluence on glass slides. To remove the EC, the veins were cannulated, rinsed with 100 mL phosphate-buffered saline (PBS) and then filled with 0.03% collagenase in PBS and incubated for 30 minutes at room temperature. After incubation, the enzyme solution was flushed through the cord with 100 mL PBS, and the effluent was collected and centrifuged at 100 g for ten minutes. The cell pellet was resuspended in medium 199, supplemented with 20% FCS and antibiotics, and seeded onto 75 × 38-mm glass slides (Fisher, Medford, MA) that had been pretreated with 0.5 mol/L NaOH for two to three hours and rinsed. Cultures were incubated at 37°C and became confluent after three to four days. Experiments were conducted within four days after the cultures reached confluency. Identification of the monolayer as EC was obtained by positive immunofluorescent staining for factor VIII-related antigen and by morphological assessment. Viability was assessed by trypan blue exclusion.

RBC-EC adherence assay. EC monolayers on glass slides formed the base of a modified Richardson flow chamber that was mounted in a video microscopy system on the microscope stage (Fig 1). The chamber depth was controlled by the thickness of the gasket, and for the experiments presented here, was machined to either 170 μm or 104 μm. RBC suspensions at a 1% hematocrit were perfused into the chamber for five to ten minutes at a constant flow rate (wall shear rates varied from 25 to 500 s⁻¹) followed by a 20-minute rinse with cell-free suspending medium at the same flow rate. In some experiments, a ten-minute incubation period without flow preceded the rinse, during which time the RBC were allowed to settle onto the EC monolayer. Perfusion was achieved with a Harvard syringe

pump. The red cell suspensions were maintained in a 37°C water bath, and the chamber was maintained at 37°C by an air curtain incubator. The number of adherent RBC was determined at 1, 10, and 20 minutes of rinse for the same single microscope field. After 20 minutes of rinse, multiple microscope fields were examined. The initial count was made after static incubation and a one-minute period of rinsing to remove nonadherent settled cells. In the continuous-flow experiments where the incubation period was eliminated, the rinse immediately followed the RBC perfusion, and the number of adherent RBC was determined at ten and 20 minutes for the same microscope field. A video camera (RCA TC 1005; Lancaster, PA) mounted to a Nikon Diaphot microscope was used to record and display experiments. Experiments were recorded on video tape (Gyr TL 2001; Anaheim, CA) for later playback and analysis and displayed on a high-resolution TV monitor.

The wall shear stress in the flow chamber was calculated by using the momentum balance for a newtonian fluid and assuming a parallel-plate geometry and fully developed flow. In easily measurable experimental quantities, it is given by the formula $\tau = 3\mu Q / 2a^2b$ dynes/cm² (wall shear stress) where Q is the flow rate, cm³/s; μ is the fluid viscosity, dynes · s/cm²; $2a$ is the gap width (chamber depth), cm; τ is the wall shear stress, dynes/cm²; and b is the slit width, cm.

The wall shear rate ($\dot{\gamma}_w$) is given by $\dot{\gamma}_w = 3Q / 2a^2b$ (s⁻¹).

Velocity determination using image analysis. Taped experiments were analyzed by using a digital image processor (Model 327; Perceptive Systems Inc, Houston) for image quantification. To determine the velocity of individual cells, sequential images separated by a given delay time were digitized from tapes. The position of a particular red cell was noted in each digitized frame, and the velocity was determined from the number of pixels (picture elements into which a digitized frame is divided) a cell had moved between frames for the given delay time. All velocities were converted to micrometers per second by using a calibration on the microcomputer. Velocity measurements were made during the cell perfusion before the rinse cycle was initiated. At least 200 cells were tracked at each shear rate and results presented as a velocity histogram. For a given video frame, all nonadherent cells in focus near the EC surface were automatically tracked and entered into the histogram.

Statistical methods. The data of multiple microscope fields after 20 minutes of rinse were analyzed by using an f test that distinguishes between the treatment variance (different blood samples, eg, HbAA, HbSS, HbAA [high-reticulocyte], or HbAS sheared) and the variances due to the random or uncontrolled variables (different donors, different EC monolayers, field-to-field variances within a monolayer, etc). For comparisons of several single field measurements at different times (1, 10, and 20 minutes of washout) the Student's t test was used.

RESULTS

When examined under flow conditions after a static ten-minute incubation period, sickle RBC were more adherent to EC than normal RBC (Table 1). A larger fraction of settled RBC remained adherent for SS RBC (1%) than for AA RBC (0.16%) after one minute of rinsing. These percentages are the ratio of adherent cells after one minute of rinse (Table 1), divided by the cell density of the initial monolayer of RBC in contact with the endothelium after static incubation, and multiplied by 100. RBC from individuals with AS did not differ significantly from normal RBC in their adhesion to EC. Mechanically traumatized (sheared) RBC, both normal and sickle trait, were also more adherent than normal RBC, but not as adherent as sickle RBC. Results from

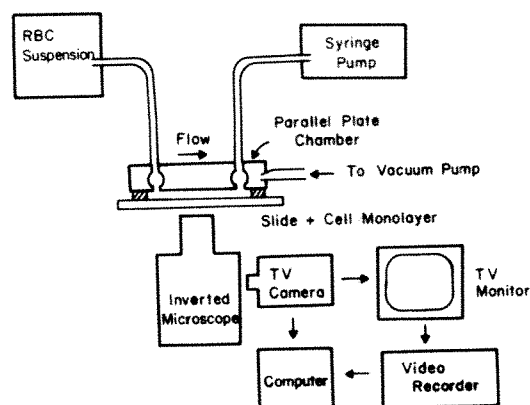


Fig 1. Schematic diagram of the adherence apparatus consisting of a flow chamber with a rectangular Silastic gasket and a glass slide with the attached endothelial monolayer. These are held together by a vacuum maintained at the periphery of the slide, forming a channel of parallel-plate geometry. The height of the flow channel (gap width) is controlled by the thickness of the gasket. RBC suspensions and culture medium enter and exit the channel through two slits machined in the polycarbonate plate. Flow is controlled by using the Harvard syringe pump. The chamber is mounted on an inverted microscope equipped with a video camera. For chambers used in these experiments, the range of wall shear rates possible is 0 to 5,000 s⁻¹, covering most of the range of physiological interest.

Table 1. Adhesion of Normal, Sickie, Sickie Trait, and Sheared RBC to EC at a Wall Shear Stress of 1 dyne/cm²

RBC	No. of Donors	Adherent RBC/mm ² at Rinse Times		
		1 min	20 min	Multiple Fields at 20 min
AA (n = 19)	8	24.78 ± 16.46	8.11 ± 5.28	6.62 ± 6.10 (s = 284)
SS (n = 13)	4	165.70 ± 142.62	52.56 ± 44.08	39.32 ± 29.72 (s = 211)
AS (n = 2)	1	10.41	8.33	9.25 ± 8.25 (s = 32)
AA sheared (n = 10)	6	40.83 ± 11.42	17.08 ± 8.66	22.25 ± 13.46 (s = 165)
AS sheared (n = 2)	1	29.16	16.66	17.16 ± 9.04 (s = 32)

NOTE. In these experiments, there was a ten-minute incubation period before the rinse was begun. The flow chamber gap width was 170 μm . The data at the one- and 20-minute rinses represent the means of single fields counted in *n* experiments. The data in the far right column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse (*s*, total fields in all *n* experiments). The differences between the AA controls and the SS, AA sheared, and AS sheared RBC had a level of significance of $P < .01$ (*f* test for multiple fields at 20 minutes and Student's *t* test for single fields at 20 minutes) after 20 minutes of rinse. There was no significant difference between the adhesion of AA and AS RBC.

experiments performed using RBC from a splenectomized patient with HbAA indicated that these RBC behaved like normal cells with respect to adhesion to EC (data not shown).

Experiments on density-separated sickle, normal, and high-reticulocyte control RBC revealed that the least-dense fraction (fraction with the highest percentage of reticulocytes) was more adherent than the denser fractions and the unfractionated sample (Table 2). The high-reticulocyte control RBC were more adherent than normal, which suggested that the degree of adhesion may be partially related to the percentage of young cells in the cell population.

The same differences in adhesion between the RBC populations were noted when the ten-minute incubation period was eliminated so that the entire experiment was conducted under flow conditions (Table 3). Under conditions of continuous flow a smaller chamber gap width was utilized, thereby reducing the total flow area and increasing the potential for RBC-EC interactions. In the data presented in Table 3, the flow rate was decreased so that the smaller gap width maintained the same wall shear stress of 1 dyne/cm² (wall shear rate of 100 s⁻¹).

To determine the dependence of sickle and normal RBC adherence to EC on the wall shear rate, experiments were performed at shear rates ranging from 25 s⁻¹ to 500 s⁻¹. The RBC adherence after the 20-minute rinse period as a function of wall shear rate is plotted in Fig 2. For both sickle and normal RBC, adhesion decreases as shear rate increases.

Little or no adhesion is observed after 20 minutes of washout at shear rates exceeding 170 s⁻¹ for normal RBC and at shear rates exceeding 260 s⁻¹ for sickle RBC. The difference between normal and sickle cell behavior with respect to adhesion appears to be more pronounced at the lower shear rates.

Typical velocity distributions of sickle and normal RBC obtained from video tapes at two specific wall shear rates are plotted in Figs 3 and 4 in terms of the percentage of total nonadherent RBC in the frame traveling in a particular velocity range. At each shear rate (flow rate), it is apparent that a higher proportion of sickle RBC are traveling at slower velocities—whereas a higher proportion of normal RBC are traveling at the higher velocities. Sickle cells move more slowly across the endothelial surface than normal cells. The average velocity of all of the near-wall nonadherent cells in a given frame was usually at least twice as high for normal RBC as it was for sickle RBC at the same bulk volumetric flow rate.

DISCUSSION

Shear rate plays an important role in determining the rheological interactions of sickle cells in the circulation, particularly at the small vessel level (arteriole, capillary, venule). In cell filtration studies, the relative resistance to flow offered by both oxygenated and deoxygenated SS cells is much lower when the flow velocity is high.¹⁸ At low shear

Table 2. Effect of Cell Density on Normal and Sickle Cell Adherence to EC After a 20-Minute Rinse at a Wall Shear Stress of 1 dyne/cm²

	SS			AA (High-Reticulocyte)		AA	
	ISC (%)	Reticulocytes (%)	Adherent RBC*	Reticulocytes (%)	Adherent RBC*	Reticulocytes (%)	Adherent RBC*
Whole	16.9	16.1	87.75 ± 17.88	8.4	38.54 ± 19.54	0.8	6.25 ± 3.42
Top	9.6	33.2	92.42 ± 31.58	15.0	48.96 ± 13.83	3.3	11.17 ± 6.54
Middle	22.6	6.7	52.63 ± 11.67	6.0	22.13 ± 6.92	0.6	5.71 ± 2.58
Bottom	28.6	8.7	51.29 ± 13.29	1.0	22.92 ± 7.88	0.6	8.83 ± 4.00

NOTE. In these experiments, there was a ten-minute static incubation period before the rinse was begun. The flow chamber gap width was 104 μm . The data are from a representative experiment for each RBC type. AA (high-reticulocyte) RBC were obtained from an iron-deficient patient responding to treatment. The differences between normal AA and the SS and AA (high-reticulocyte) RBC had a level of significance of $P < .0005$ (using the *f* test) for whole blood samples. For fractionated samples, there was no significant difference between the adhesion of middle and bottom cells, but the differences between the top and middle and the top and bottom cells had a level of significance of $P < .0005$ for SS and AA (high-reticulocyte) RBC.

Abbreviation: ISC, irreversible sickled cells.

*Average number of adherent RBC/mm² ± SD per microscope field (16 fields counted after a 20-minute rinse).

Table 3. Adhesion of Normal, Sickie, and Sheared Red Cells to EC Under Conditions of Continuous Flow

RBC	Adherent RBC/mm ² at Rinse Times		
	10 min	20 min	Multiple Fields at 20 min
AA (n = 12)	14.77 ± 6.49	11.36 ± 8.16	11.48 ± 6.83 (s = 176)
SS (n = 12)	88.98 ± 22.56	59.09 ± 35.08	45.12 ± 26.76 (s = 164)
AA (sheared) (n = 6)	22.92 ± 9.85	20.14 ± 6.55	20.65 ± 7.80 (s = 95)
AA (high reticulocyte)* (n = 5)	80.46 ± 32.64	49.81 ± 18.82	43.71 ± 23.59 (s = 76)

NOTE. In these experiments, there was no static incubation period before the rinse was begun. The flow chamber gap width was 104 μm , and the wall shear stress was 1 dyne/cm². The data at the ten- and 20-minute rinses represent the means of single fields counted in n experiments. The data in the far right column reflect the sum of multiple fields counted in each experiment after a 20-minute rinse (s, total fields in all n experiments). The differences between the AA control and the SS, AA sheared, and AA (high-reticulocyte) RBC all had a level of significance of $P < .0005$ (using the *t* test) for multiple fields at 20 minutes. For single fields at 20 minutes using the Student's *t* test, $P < .0005$ for AA v SS and AA v high-reticulocyte AA, whereas $P < .025$ for AA v sheared AA.

*AA (high-reticulocyte) RBC were obtained from two donors, a patient responding to treatment for iron deficiency and a patient with hereditary spherocytosis.

rates, significant problems may develop in the microcirculation due to obstructed flow caused by sickle cells becoming stiff and/or by SS cells interacting with EC. In a recent report, Rodgers et al¹⁹ presented evidence that microcirculatory flow in patients with sickle cell disease is periodic. The increased adhesion of sickle cell RBC to microvascular or venule endothelium may be a rheological factor that contributes to local flow disturbance and induces oscillation. On the other hand, the high local shear stresses obtained in oscillatory flow could facilitate the passage of red cells through small vessels by helping them to deform and may dislodge sickle cells already adherent to the endothelium. Less-dense sickle RBC, which appear to be more adherent than heavier cells, may play a role in initiating vasoocclusion by sufficiently retarding microvascular flow to extend the transit times of more-dense upstream cells beyond the delay time required for gel formation. This phenomenon may be particularly important in the venules where the chances for adhesive events are increased because of the low shear rates involved and where cell transit times may be approaching the pregelation times for heavier sickle cells. In this case, extending transit times even slightly could result in increased

polymer content and sickling. Recent reports have shown that polymer fraction may be correlated with vasoocclusive severity,²⁰ though not in all cases.²¹ Whether interactions between sickle erythrocytes and vascular EC are significant enough to play a role in the obstruction of flow would be influenced by the wall shear rate. Results from our studies suggest that adherence of sickle cells is enhanced at low shear rates but is not very different from that of normal RBC at high shear rates (where long-term adherence to endothelium for all RBC is very low).

Digital image analysis techniques were used to analyze videotaped flow experiments to gain insight into dynamic RBC-EC interactions by determining the velocities of individual sickle and normal RBC. The velocity of cells plays an important role in the promotion or inhibition of cell adhesion. Because of the inward radial migration and wall exclusion of RBC, which are important in flow channels whose height is many times that of the red cell, most of the RBC in a suspension will be flowing at relatively high speed in the core of the channel. Erythrocytes that are traveling in the cell-poor region near the wall are actually moving at different rates that, in some cases, may be slow enough to allow adhesive interactions to take place between the RBC and the

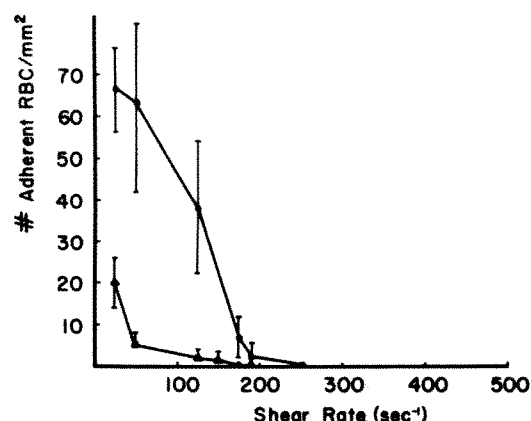


Fig 2. Effect of shear rate on adherence of sickle and normal RBC to EC after 20 minutes of washout at a fixed wall shear rate. The chamber used for these studies was the same as described in Table 3, and all experiments were done under continuous flow (no incubation). Data points are means with SD. (●), SS; (▲), AA.

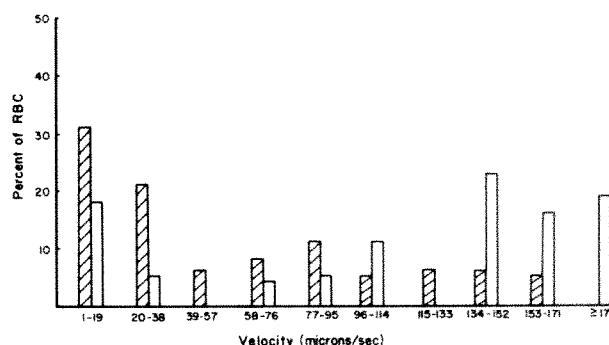


Fig 3. Velocity distribution for sickle and normal RBC near the EC monolayer at a wall shear rate of 126 s⁻¹. Cells moving at less than 1 $\mu\text{m/s}$ were not included in the distribution because they were considered to be adherent. More than 200 individual cell velocities were obtained for both SS and AA samples. The average velocities were 60.2 $\mu\text{m/s}$ for SS RBC (▨) and 121.7 $\mu\text{m/s}$ for AA RBC (□).

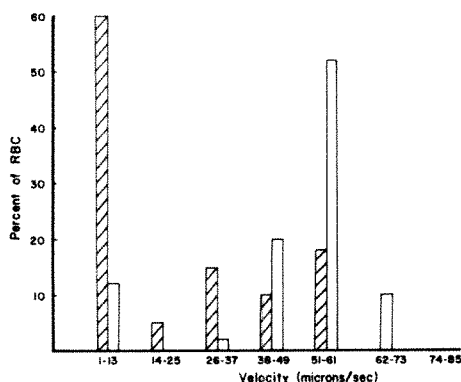


Fig 4. Velocity distributions for sickle and normal RBC at a wall shear rate of 50 s^{-1} . The flow chamber and number of cells tracked were the same as in Fig 3. The average velocities were $14.2 \mu\text{m/s}$ for SS RBC (▨) and $43.4 \mu\text{m/s}$ for AA RBC (□).

EC lining the vessel wall. From qualitative observations of videotaped experiments, sickle RBC traveling near the EC surface appeared to be moving more slowly than normal RBC near the EC surface for a given bulk volumetric flow rate. Quantitative velocity measurements presented earlier utilizing digital image processing confirmed these observations. Some red cells (particularly in the case of sickle RBC) appeared to be sliding slowly across the endothelial surface as if temporary contacts between the cell surfaces were being made but were not strong enough for the red cell to adhere. Differences in the adherence properties of sickle and normal RBC (sickle RBC may be more "sticky") could account for the slower velocities of sickle RBC near the EC monolayer.

The increased adhesion of less-dense normal and high-reticulocyte control RBC suggests that the increased adhesive properties of SS RBC may be, at least in part, a reflection of their young mean age. However, other factors may be involved. Mechanical damage of the red cell can also lead to increased RBC/EC adhesion as shown by our results with sheared normal RBC (Tables 1 and 3). Several investigators have shown that sickle RBC are much more susceptible to shear damage than normal cells^{22,23} and that hemolysis can occur at stress levels that are obtained physiologically. Sublytic mechanical damage to sickle cells in the circulation may also predispose them to increased EC adherence.

Exposure of normal RBC to subhemolytic shear stresses is known to induce a deformability defect and lead to calcium accumulation without alterations in morphology.¹⁴⁻¹⁶ After sublytic shear stress exposure, both AA and AS RBC were

more adherent to EC than normal RBC. Further study of these stressed cells may aid in determining the characteristics of the SS RBC that make them more adherent.

Plasma factors have been shown to influence sickle RBC adhesion.^{3,5} Results obtained in this study indicate that adhesion differences between erythrocytes can be demonstrated in the absence of specific human plasma factors peculiar to SS plasma because these experiments utilized washed red cells and a tissue culture suspending medium. Specific SS plasma factors may play a modulating role in the adhesion process but were not necessary to demonstrate altered adhesion in sickle RBC in our system.

Previous experimental evidence has suggested at least two phenomena that may predispose to vasoocclusion in sickle cell disease: (a) intracellular gel formation, which leads to shape change and decreased deformability of SS RBC,²⁴ and (b) increased adhesion of SS RBC to EC.¹⁻⁶ In this study, the adhesion process was examined under controlled fluid mechanical conditions to gain insight into events that may lead to vasoocclusion but do not directly involve the deformability of the SS RBC. Cells with maximum intracellular gel formation and shape change are the most-dense cells,^{25,26} whereas our studies indicate that the most-adhesive cells are the least-dense RBC. Although the least-dense cells are hemodynamically the most competent in deoxygenated blood,²⁶ their tendency to adhere could sufficiently retard flow in the small vessels to extend transit times for other cells beyond the critical delay time required for gel formation. The delay time for HbSS gelation is strongly dependent on the intracellular hemoglobin concentration. Thus, dense cells with high mean corpuscular hemoglobin concentrations may have a profound effect on the microcirculatory flow if their capillary transit times are increased. The adhesion of light (young) cells may lengthen transit times for other cells in the microvasculature, thereby leading to gel formation in dense cells and resulting in vasoocclusion. Recently, increased adhesion of reticulocytes in SS disease to fibronectin-coated Petri dishes has been demonstrated in a static system.²⁷ The abnormal adherence of young cells may be of little or no consequence in other diseases that are characterized by increased numbers of reticulocytes but do not involve vascular occlusive events. Increasing capillary transit times would not result in changes in the rheological properties of RBC for these diseases. Increased adherence to endothelial cells, the only factor besides delay time thought to be correlated with disease severity, may be an important feature of young RBC in sickle cell disease.

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Erythrocyte Membrane Skeleton Abnormalities in Severe β -Thalassemia

By E. Shinar, O. Shalev, E.A. Rachmilewitz, and S.L. Schrier

The protein composition of ghosts, inside-out vesicles (IOV), and membrane skeletons (MS) of erythrocytes (RBC) from splenectomized (spx) and nonsplenectomized (non-spx) patients with β -thalassemia major and β -thalassemia intermedia was determined. Ghosts from spx thalassemia intermedia patients had a significant increase in their globin content (which was mostly heme reactive) and contained extra polypeptides in the protein 4.2 to 5 and 6-globin areas. The Triton-extracted MS from all of the thalassemic patients showed two major abnormalities: (a) they retained up to twice the amount of protein 3 when compared with controls; (b) they had a significant increase in their globin content, the concentration of which was independent of their protein 3 content. Analysis of the IOV revealed no differences between those prepared from

normal controls and those of the patients. MS from spx thalassemia intermedia patients were grossly abnormal when examined by scanning electron microscopy and they exhibited aggregates of material that on transmission electron microscopy suggested the presence of globin precipitates. We propose that, although the integral protein composition, as reflected in the IOV, from severely affected β -thalassemics is intact, their MS assembly is deranged. The altered skeletal structure of thalassemic RBC could result from attachment of denatured globin to the skeleton components. These abnormalities may contribute to the premature cell death seen in severe β -thalassemia.

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THE THALASSEMIA are a group of inherited disorders in which globin chain synthesis is impaired. Although the genetic defects of β -thalassemia have been studied intensively¹ the reasons leading to the premature removal of the abnormal RBC in the marrow and from the peripheral circulation are not clearly understood. It has been proposed that the extra, unmatched α -globin chains precipitate in the RBC, resulting in alteration of normal structure, function, and deformability of the membrane.² These changes, when expressed at the RBC exterior, perhaps by reverse transmembrane signaling, might indicate to the macrophagic system that these cells should be removed. Indeed modification of the external membrane surface has recently been found.³ There is also evidence of more widespread membrane dysfunction in β -thalassemia. Morphological changes in the membranes of thalassemic RBC have been documented in ultrastructural studies of mature as well as nucleated cells.^{4,5} Moreover, membranes of thalassemic RBC are known to be more sensitive to oxidant stress, as reflected by an increase in malonyldialdehyde formation, a decrease in polyunsaturated fatty acid levels,⁶ an increase in protein cross-linking,⁷ and a decrease in titratable membrane thiols.⁸

In this study we tried to identify alterations of the β -thalassemic RBC membrane that might be produced by precipitation of excess unmatched α -globin chains at the

cytosolic surface of the membrane. Accordingly, we analyzed the polypeptide composition of isolated hypotonic white ghosts from severely affected but untransfused patients with β -thalassemia intermedia and transfused patients with β -thalassemia major. We then resolved the membrane into its component parts by studying its peripheral protein membrane skeleton (MS), as reflected in Triton-extracted cytoskeletal shells,⁹ and the integral membrane proteins, as reflected in inside-out vesicles (IOV) prepared from the same RBC.

MATERIALS AND METHODS

Patients Studied

Samples of heparinized venous blood were collected from a total of 18 patients with β -thalassemia intermedia, five patients with β -thalassemia major, five patients with other hemoglobinopathies, and four nonhemoglobinopathic reticulocyte-rich patients as well as 14 normal splenectomized and nonsplenectomized subjects under protocols approved by the Hadassah Hospital Committee on Human Experimentation and the Stanford Committee on Human Experimentation. The majority of the thalassemic patients were of either Kurdish-Jewish or Israeli-Arabic extraction. Eleven of the 18 patients had been splenectomized (spx) several years prior to the present study whereas the rest were nonsplenectomized (non-spx). Initially samples were studied in Jerusalem within 24 hours after blood collection. In order to confirm reproducibility, to apply more stringent inhibition of proteolysis, and to utilize newer methods of quantifying polypeptide composition, most patients were restudied at Stanford. Heparinized whole blood samples arrived at Stanford within 36 hours after being drawn. Upon arrival they were washed, and the buffy coat was removed. The hematocrit value was brought to 50% with isotonic saline, to which 2 mmol/L diisopropyl fluorophosphate (DFP) and 10 μ g/mL of pepstatin A and of leupeptin were added to prevent proteolysis. The thalassemia intermedia patients rarely required blood transfusions, and none had been transfused 2 to 3 months prior to study. The thalassemia major samples were obtained just prior to the next transfusion, typically 4 weeks after the last transfusion. Each sample was not subjected to all of the analyses eventually used. The detailed hemoglobin and globin chain synthesis ratios of most of the patients have been previously reported.¹⁰

Preparation of Ghosts and Membrane Fractions

White ghosts were prepared by lysis of RBC in hypotonic phosphate buffer according to the method of Dodge et al.¹¹ The lysis

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buffer contained 1 mmol/L EDTA and 0.1 mmol/L phenylmethylsulfonyl fluoride to inhibit protease activity (0.2 mmol/L DFP was used for the shipped samples). Ghosts prepared from the spx thalassemia intermedia patients retained a brownish color despite extra washes with 40-fold volumes of lysis buffer. IOV were prepared by incubating white ghosts in 0.5 mmol/L sodium phosphate buffer (pH 8) at 0°C for one hour and then harvesting them at the interface of a Dextran T-500 barrier (Pharmacia Fine Chemicals, Piscataway, NJ) by centrifugation in a Spinco SW41 rotor (Spinco-Beckman, Palo Alto, CA) for two hours at 40,000 rpm.¹² MS were prepared by direct lysis of intact RBC (pretreated both in Jerusalem and Stanford with 2 mmol/L DFP, 10 µg/mL pepstatin A, and 10 µg/mL leupeptin) with Triton X-100 at a concentration of 25 mg/mL packed RBC as described by Sheetz.¹³ Since considerable care is necessary in standardizing this preparation, the Triton was weighed out, and duplicate microhematocrits were performed on the packed RBC to insure that the ratio of Triton to RBC volume was tightly controlled. The MS were extracted by using both isotonic and hypertonic 0.3 mol KCl buffer. To control proteolysis further the extraction buffers and sucrose gradients contained 0.25 mmol/L DFP, 10 µg/mL pepstatin A, and 10 µg/mL of leupeptin. The addition of hypertonic KCl removed considerably more integral proteins and produced MS containing substantially less protein 3.¹³ The amount of protein 3 retained in these MS was calculated as a percentage of the total protein after subtracting the globin and protein 6-globin content in each sample to provide a more equivalent denominator for comparative purposes. On several occasions, cellulose fiber columns were used to remove the WBC and platelets.¹⁴ No differences in the results were found when this time-consuming step was used, and therefore it was not used routinely.

When it became apparent that MS from spx β -thalassemia intermedia RBC contained more globin than the non-spx MS, it was important to determine whether that globin was firmly adherent to the MS or simply reflected aggregates of globin enmeshed or trapped in the cytoskeleton at the time of Triton skeleton formation. To explore this possibility, MS from spx β -thalassemics were frozen once at -20°C and then thawed by warming up to room temperature slowly. Aliquots were set aside, and the remainder was sonicated for three to five minutes at output control 3 in the probe sonicator Ultrasonics, Inc. Model W-220 (Orangeburg, NY). All samples were then recentrifuged at 100,000 g for 60 minutes, and the pellets were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as will be described later.

Protein Determination

Protein was determined by the Bradford method,¹⁵ with bovine serum albumin as a standard, and by the method of Lowry et al.¹⁶ In an attempt to distinguish free globin from heme-containing globin, the hemoglobin content of the several membrane preparations was determined in Jerusalem by using the benzidine reaction.¹⁷

SDS-PAGE

Samples were separated on 6% to 18% nonlinear gradient slab gels according to the method of Laemmli.¹⁸ The gels were stained with Coomassie blue or PAS reagent. In Jerusalem the gels were scanned and analyzed in an automated standardized Helena Quick Quant II densitometer (Helena Laboratories, Beaumont, TX). The area occupied by each band was determined with a recording integrator. The protein composition of the gels of the samples studied at Stanford was determined by the method described by Agre et al.¹⁹ Briefly, Coomassie blue-stained bands were cut out, eluted with 1 mL of 25% pyridine, and the absorbance at 605 nm was measured. This method yielded reproducible quantitation of the spectrin:protein 3 ratios and supported the densitometric determinations.

Immunoreactive Spectrin

To determine whether some of the polypeptide bands that appeared in excess in the thalassemic ghosts and MS were products of proteolytic attack on spectrin we used the technique of Western immunoblotting. Proteins from SDS-PAGE were transferred to nitrocellulose paper exactly as described by Towbin et al.²⁰ Then after unoccupied sites were covered with albumin, the nitrocellulose was reacted with our polyclonal-monospecific rabbit antispectrin antibody,²¹ and the sites of binding were identified by the immunoperoxidase reaction.²² Screening studies had established that with the use of this technique our monoclonal mouse antispectrin antibody reacted only with β -spectrin,²³ whereas the rabbit polyclonal antibody reacted only with α - and β -spectrin at a ratio of roughly 80:20. These studies were done only on the samples sent to Stanford.

Electronmicroscopic Studies

Both white ghosts and MS were examined by transmission electron microscopy (TEM) in a Philips EM-300 electron microscope (Philips, Mahwah, NJ) after fixing with a 2% solution of glutaraldehyde in phosphate-buffered saline and then embedding and sectioning.²⁴ Studies by the scanning electron microscope (SEM) were performed after processing of the samples according to the method of Sanders et al.²⁵ using a Jeol-35 scanning electron microscope (Jeol USA, Inc. Peabody, MA).

Statistical Analysis

Statistical analysis was carried out using the Abstat program (Abstat-Anderson-Bell Co, Canon City, CO) for the IBM-PC (IBM, Boca Raton, FL).

RESULTS

Polypeptide Analysis

Hypotonic ghosts. In the spx thalassemia intermedia ghosts studied at Stanford there was an increase in the globin content that reached $13.7\% \pm 3.5\%$ (mean $\pm$ S.D.) of the total amount of protein compared with $3.0\% \pm 1.6\%$ and $4.7\% \pm 2.7\%$ in the non-spx thalassemics and normal controls, respectively ($P < .0001$) (Table 1, Fig 1). Studies performed in Jerusalem yielded similar results ($8.9\% \pm 0.7\%$, $3.7\% \pm 0.2\%$, and $2.9\% \pm 1.0\%$, respectively). The benzidine reaction performed on the samples in Jerusalem showed that the majority of this membrane-associated globin was heme containing ($8.2\% \pm 4.4\%$, $3.8\% \pm 2.8\%$, and $2.9\% \pm 1.2\%$, respectively). In addition, extra polypeptides appeared in the ghosts from spx thalassemia intermedia patients in the areas between proteins 4.2 to 5 and 6-globin. The increase in the 4.2 to 5 area was significantly greater in spx patients than in the comparable controls ($P = .03$), but there was no significant difference between controls and non-spx thalassemics ($P = .3$). The increase in the 6-globin area in spx patients was significantly greater than either controls ($P = .0003$) or non-spx thalassemics ($P = .0006$); however, there was no significant difference between controls and non-spx thalassemics ($P = .26$). Although the changes in polypeptide pattern in the 4.2 to 5 area and the 6-globin area seem to be present in other hemoglobinopathies as well, the increase in globin content was highest in the spx β -thalassemics. The spectrin:protein 3 ratio was slightly lower in the spx thalassemics than in either

Table 1. Analysis of SDS-PAGE of Ghosts of Thalassemia Intermedia Patients and Controls

	Spectrin/ Protein 3 Ratio	Globin*	4.2-5*	6-Globin*
Normal controls (6)	1.35 ± .10	4.7 ± 2.7	9.5 ± 2.1	5.7 ± 1.3
Thalassemia intermedia, non-spx (7)	1.32 ± .20	3.0 ± 1.6	10.9 ± 2.9	6.7 ± 1.7
Thalassemia, spx (11)	1.15 ± .16	13.7 ± 3.5	11.9 ± 2.0	13.0 ± 3.6
Other hemoglobinopathies				
Sickle cell anemia				
A	1.1	7.4	14.7	12.2
B	1.1	6.1	12.9	11.5
C	1.0	6.1	15.2	14.6
Hgb SC	1.4	6.2	11.3	8.2
Hgb AC	1.5	3.9	9.9	9.6

NOTE. Numbers in parentheses are the number studied.

*Expressed as a percentage (mean ± SD) of the total protein.

controls ($P = .01$) or non-spx thalassemics ($P = .05$), and there was no difference between controls and non-spx thalassemics ($P = .7$). Immunoblot analysis indicated that in the ghosts of each of the spx thalassemic patients there was a very faint band in the protein 7 area that reacted with antispectrin antibodies (data not shown). No spectrin immunoreactivity was detected in the globin, the 4.2 to 5, and the 6-globin areas.

IOV. No difference was found in the SDS-PAGE analysis of IOV obtained from thalassemia intermedia patients (spx and non-spx) and normal controls. Notably, no excess accumulation of globin was found in thalassemic IOVs (data not shown).

MS. When extracted under isotonic conditions the globin content of MS from the spx thalassemia intermedia patients was significantly higher ($11.6\% \pm 7.0\%$) than that from the non-spx thalassemia intermedia patients ($4.3\% \pm 0.7\%$) and the normal controls ($2.8\% \pm 0.5\%$) ($P < .001$) (Table 2, Fig 2). The benzidine reaction revealed

that approximately 75% ($8.8\% \pm 1.8\%$) of the globin attached to the spx thalassemic MS contained heme.

The large amount of globin in spx thalassemic MS was unanticipated, and it was therefore important to determine whether the globin was associated with the cytosol face of protein 3.²⁶ The isotonic Triton extraction leaves considerable protein 3 attached to the membrane skeleton.¹³ Therefore, RBC MS were prepared in Triton in the presence of hypertonic KCl buffer, thus producing MS containing substantially less protein 3.¹³ The increased removal of protein 3, however, was not followed by any decrease in the globin content of the thalassemia intermedia MS, which was again elevated, particularly in the spx thalassemics' MS, rising to $14.0\% \pm 5\%$ and to $7.2\% \pm 2.1\%$ in the non-spx thalassemic MS.

The globin could be firmly bound to the MS, or aggregates of cytosolic or membrane globin could have been nonspecifically enmeshed or trapped during the formation of Triton membrane skeletons. To test these possibilities MS from spx thalassemics were frozen at -20°C , thawed once, and then divided into two aliquots, one of which was saved for analysis and the other subjected to 5 seconds of sonication (see Materials and Methods). Both samples were then centrifuged and the pellets analyzed by SDS-PAGE. In one of three such experiments the globin content was initially 12.2% of the membrane protein. After freezing and thawing once,

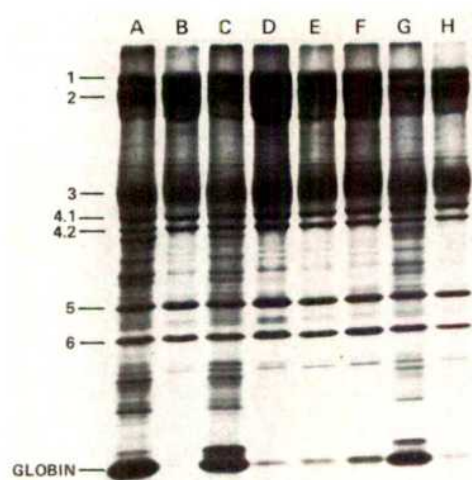


Fig 1. SDS-PAGE of RBC ghosts from three spx (lanes A, C, G) and two non-spx (B, D) thalassemia intermedia patients. Lanes E, F, and H are three normal controls. The numbers in the left-hand margin identify the protein bands.

Table 2. Globin Content of Thalassemic and Control MS Extracted Under Isotonic (I) and Hypertonic (H) Conditions

	I	H
Normal Controls	2.8 ± 0.5*	2.5 ± 1.4
Thalassemia intermedia, non-spx	4.3 ± 0.7	7.2 ± 2.1
Thalassemia intermedia, spx	11.6 ± 7.0	14.0 ± 5.0
Other hemoglobinopathies		
Sickle cell anemia		
A		3.2
B		10.6
C		4.5
Hgb SC		6.0
Hgb AC		7.1

*Globin content expressed as a percentage of the total protein (mean ± SD).

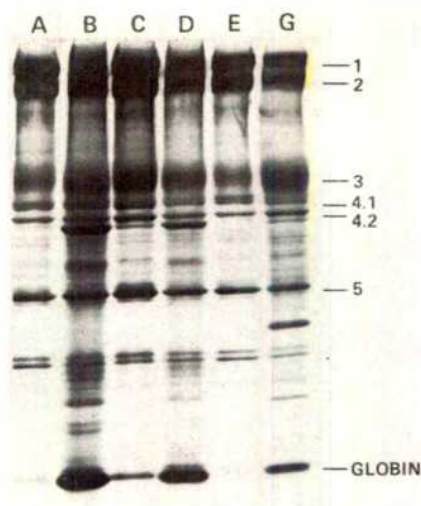


Fig 2. SDS-PAGE gels of RBC membrane skeletons extracted under hypertonic conditions. Lanes A and E are controls, B and D are spx thalassemics, and C is a non-spx thalassemic. Lane G contains normal ghosts for reference. The numbering system on the right-hand margin is the same as in Fig 1.

the globin represented 14.1% of the membrane protein, and after sonication the globin content was 13.2% of the membrane protein. The proportion of the other membrane proteins (ie, proteins 1, 2, 3, 4.1, and 5) was unchanged by either freezing and thawing or freezing, thawing, and sonication (data not shown). In performing these experiments it became apparent that under these controlled conditions the protein 3 content of thalassemic MS was increased (Fig 2). Studies were therefore extended to include five transfusion-dependent thalassemia major patients, the blood samples being obtained just prior to transfusion. The reticulocyte-rich controls were patients with hemolysis due to vasculitis (reticulocytes, 5.5%), heart transplantation (reticulocytes, 11%), chronic myeloid leukemia (reticulocytes, 6.9%), non-spx hereditary spherocytosis (reticulocytes, 15%), and diffuse histiocytic lymphoma (reticulocytes, 7.8%). The values for the protein 3 content of MS in normal controls and five reticulocyte-rich subjects are not different (Table 3). Three patients with sickle cell anemia whose reticulocyte counts ranged from 10.4% to 21.4% were also studied. The protein 3 content of their MS were comparable in each case to the

reticulocyte-rich control values (Table 3). Spx and non-spx thalassemia intermedia patients have similar values and are therefore presented together. There are no significant differences in the protein 3 content of MS prepared from thalassemia intermedia and thalassemia major patients. However, the elevation of the protein 3 content in thalassemics is significant, and the differences between controls and thalassemia intermedia patients and between controls and thalassemia major patients have *P* values of .0009 and .0015, respectively. In addition, MS from spx thalassemic patients retained small amounts of the glycophorins detected by PAS staining. Control MS were PAS-negative as previously shown by Sheetz¹³ (data not shown).

Morphological Changes

When thalassemic MS were analyzed by using both SEM and TEM, significant morphological changes were seen in MS from the spx patients as compared with the normal controls. The SEM typically showed a fine network in normal MS that was grossly distorted in all the spx thalassemic MS (Fig 3). The morphological changes consisted of a thick and coarse mesh that contained large excrescences. When analyzed by TEM (Fig 4), these bumps were seen to consist of electron-dense material that was unevenly distributed in the spx thalassemic MS and had an appearance similar to that described for Heinz bodies.

DISCUSSION

The β -thalassemic RBC provides an example wherein events inside red cells lead to the premature removal of affected RBC. We postulate that the unmatched α -globin aggregates lying at the membrane cytosolic face cause an alteration transmitted to the external membrane surface that eventuates in macrophagic attack and removal of the affected cells. The signal to the macrophage might be complex. There is known to be a decrease in thalassemic membrane sialic acid residues that is unevenly distributed.⁷ It has been recently shown that after the removal of the sialic residues there is exposure of the underlying galactosyl residues to which the naturally occurring antigalactosyl antibodies bind,³ thus providing an Fc signal to macrophages. Reduced RBC deformability has also been described in thalassemia,²⁷ and such a change could, by prolonging the contact between damaged RBC and the macrophage, result in greater opportunity for macrophagic attack. RBC rigidity in thalassemia is not caused by an increased mean corpuscular hemoglobin concentration or by a reduced surface area-to-volume ratio but is most likely caused by an altered membrane modulus.²⁸ This change in membrane function could also be caused by the accumulation of α -globin aggregates at the membrane cytosol face that affect either integral membrane or MS proteins or both.

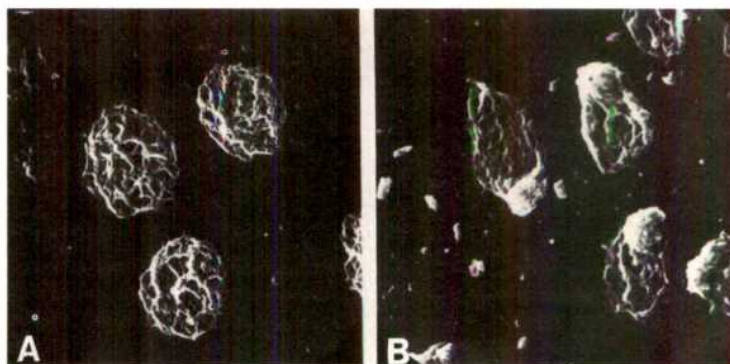
In this study as in prior studies,^{29,30} SDS-PAGE analysis of RBC ghosts from spx thalassemic patients revealed an increase in their globin content as compared with the non-spx patients and normal controls (Table 1, Fig 1). In addition, extra polypeptides in the protein 4.2 to 5 and 6-globin areas appeared in the ghosts from the spx thalassemia intermedia RBC and were free of spectrin degradation products as

Table 3. Protein 3 Content of MS Prepared in the Presence of Hypertonic KCl

	Protein 3 (%)
Normal subjects (8)	12.6 $\pm$ 1.8
Reticulocyte-rich patients (5)	9.9 $\pm$ 2.2
β -Thalassemia intermedia (4 spx, 3 nonspx)	16.6 $\pm$ 1.8
β -Thalassemia major (5)	17.9 $\pm$ 2.7
SS-1	8.6
SS-2	9.9
SS-3	7.2

NOTE. Values are calculated as a percentage of the total membrane protein less the globin and protein 6-globin content (mean $\pm$ SD). The numbers in parentheses are the number of subjects studied.

Fig 3. Scanning electron micrograph of RBC membrane skeleton from a normal control (A) and a spx thalassemic patient (B). (Original magnification $\times 6,000$.)



detectable by the Western immunoblot technique. The appearance of these extra polypeptides occurred despite the regular use of comprehensive antiproteolysis measures. These polypeptides could result from attachment of cytoplasmic proteins such as catalase to the thalassemic membranes.

Alternatively some of these bands could represent dimers, trimers, or even tetramers of native or degraded hemoglobin. Allen and Cadman³¹ have shown in an in vitro model that an increase in calcium uptake by RBC induces similar changes in their membrane polypeptides. We have recently demonstrated that there is an increased calcium content in β -thalassemia intermedia RBC that was most pronounced in the spx patients.³² It is therefore possible that calcium plays an important role in thalassemic RBC that is apparent despite the rigorous antiproteolysis measures taken. We compared the changes in spx thalassemic ghosts with the ghosts from patients with other hemoglobinopathies. The increase in polypeptides in the 4.2 to 5 and 6-globin areas seems to be nonspecific. However, the elevation in globin content in the spx thalassemic is considerably greater than the values seen in three patients with sickle cell anemia who are thought to be hyposplenic by conventional clinical criteria.

Resolution of membranes into their integral proteins (ie, IOV) and MS components revealed that there were no differences in IOV from thalassemia intermedia patients (spx or non-spx) and controls. The absence of globin from IOVs prepared from spx thalassemia intermedia patients suggested either that the globin was not attached to integral

proteins or lipids or that it could be removed by low-ionic strength media. Surprisingly it was the MS from the spx thalassemia intermedia patients that contained significantly increased amounts of globin at a level that was approached by only one of the three sickle cell anemia patients studied (Table 2). This globin could not be removed by fairly drastic physical means consisting of freezing and thawing with or without subsequent sonication. The proportion of other membrane proteins, particularly protein 3, was also not altered by these treatments. Therefore it is likely that the globin and other perhaps associated membrane constituents are firmly bound to membrane skeletons. Protein 3 is known to be one of the membrane binding sites for hemoglobin²⁶ and for hemichromes.³³ Therefore we used hypertonic KCl to increase the Triton extraction of protein 3 from thalassemic MS. However, this maneuver was not followed by any decrease in globin content (Table 2). Thus it is possible that cytoskeletal proteins serve as an attachment site for globin aggregates. Snyder et al³⁴ reported that during in vivo senescence there is increased binding of globin chains to spectrin, and it has been hypothesized that it is the α -chain of hemoglobin that binds to the α -chain of spectrin.³⁵ However, our data do not identify the site(s) of attachment of the putative α -globin aggregates to the membrane skeleton. The idea that association of globin aggregates to MS components occurs in spx thalassemics is further supported by the distinctly abnormal morphology of the MS from spx thalassemics. On SEM the MS from spx thalassemic intermedia patients displayed coarse stranding and bumps (Fig 3), and on conventional TEM, the bumps were aggregates of unevenly scattered electron-dense material. The possibility that these inclusion bodies, originally thought to be Heinz bodies, represent α -globin aggregates is intriguing but remains to be proved.

In the course of performing experiments using hypertonic KCl to strip off more protein 3 from MS we observed that the level of protein 3 was increased in MS prepared from severe β -thalassemia patients (Table 3, Fig 2) but not in another hemoglobinopathy, sickle cell anemia. This increase in protein 3 was evident in both spx and non-spx thalassemia intermedia patients as well as transfusion dependent thalassemia major patients whose RBC are a mixed population. The lack of correlation between globin content and protein 3 content of the MS indicates that these are probably unrelated phenomena and that globin aggregates do not serve to link most of the protein 3 to the MS. Attachment of an



Fig 4. Transmission electron micrograph of RBC membrane skeleton from a spx thalassemic patient. (Original magnification $\times 24,500$.)

integral protein like protein 3 to the cytoskeleton could result in an increase in membrane rigidity. Chasis et al³⁶ have shown that the increased RBC rigidity produced by monoclonal antiglycophorin A antibody is associated with the attachment of glycophorin A to the MS. In fact, MS prepared from splenectomized β -thalassemic patients retain some glycophorin as indicated by PAS staining. Recent data suggest that β -thalassemic protein 4.1 has abnormal structure and function.³⁷ The retention of integral membrane proteins on the thalassemic skeleton might therefore be a consequence of abnormal interaction of protein 4.1 with both protein 3³⁸ and some of the glycophorins.³⁹

These studies are the first to explore the MS and IOVs of severely affected patients with β -thalassemia and show abnormalities in membrane skeletal assembly of their RBC. One of these changes, the increase in globin association to

MS, is most clearly seen in spx thalassemic patients, thereby suggesting that this change may define in part the most severely affected cohort of cells that are preferentially removed by the splenic macrophages. However, the increase in protein 3 and glycophorins in MS is a common finding in all severe β -thalassemic MS studied and may represent a mechanism that could serve to produce RBC rigidity. These abnormalities may be a consequence of the hemichrome-mediated oxidative potential described in thalassemics.⁴⁰ Further experiments will be needed to test these and other hypotheses.

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Development of the Human Coagulation System in the Full-Term Infant

By Maureen Andrew, Bosco Paes, Ruth Milner, Marilyn Johnston, Lesley Mitchell,
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The investigation of many hemostatic defects in the newborn is limited by the lack of normal reference values. This study was designed to determine the postnatal development of the human coagulation system in the healthy full-term infant. Consecutive mothers of healthy full-term infants born at St Joseph's Hospital in the city of Hamilton were approached for consent. One hundred eighteen full-term infants (37 to 42 weeks' gestational age) were entered into the study. Demographic information and a 2-mL blood sample were obtained in the postnatal period on days 1, 5, 30, 90, and 180. Between 40 and 79 full-term infants were studied on each day for each of the coagulation tests. Plasma was fractionated and stored at -70°C for batch assaying of the following tests: prothrombin time, activated partial thromboplastin time, thrombin clotting time, and factor assays (biologic): fibrinogen, II, V, VII, VIII, IX, X, XI, XII, and high-molecular weight kininogen. Factor

XIII subunits A and S, von Willebrand factor, and the inhibitors antithrombin III, α_2 -antiplasmin, α_2 -macroglobulin, α_1 -antitrypsin, C1 esterase inhibitor, protein C, and protein S were measured immunologically. Plasminogen, prekallikrein, and heparin cofactor II were measured by using chromogenic substrates. The large number of infants studied at each time point allowed us to determine the following: (a) the range of normal for each test at five time points in the postnatal period; (b) that coagulation tests vary with the postnatal age of the infant; (c) that different coagulation factors show different postnatal patterns of maturation; and (d) that near-adult values are achieved for most components by 6 months of life. In summary, this large cohort of infants studied consecutively in the postnatal period allowed us to determine the normal development of the human coagulation system in the full-term infant.

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THE HEMOSTATIC SYSTEM in the neonate has many unique features when compared with the adult. In general, the screening tests are prolonged at birth, the coagulation factor levels are low, and both are dependent on the gestational and postnatal age of the infant. There are published reference values for healthy infants for most coagulation factors at the time of birth; however, these data are based primarily on cord blood samples and not on samples from the infant.¹⁻¹⁶ There is minimal or no data for some components of the coagulation system during the first week of life and scarce information beyond that time. Most of the studies reporting normal values for coagulation factors in the postnatal period have used small sample sizes that allow a determination of the mean value but not an accurate assessment of the variability of the values. Thus, to date there are no reference values that allow one to determine with any degree of confidence whether a particular infant who presents with a bleeding problem has normal or abnormal values. The purpose of the following study was to determine for full-term infants the normal values for the majority of known coagulation factors and coagulation inhibitors during the first 6 months of life.

MATERIALS AND METHODS

Subjects. Healthy full-term infants (≥ 37 weeks' gestational age) born at St Joseph's Hospital in Hamilton between December 1, 1983, and March 1, 1984, were eligible for this study. Gestational age was determined based on maternal dates and the Dubowitz assessment. These infants did not experience any perinatal problems, and all received 1 mg of vitamin K intramuscularly at the time of birth, at least 12 hours prior to the first blood sample being drawn. Information regarding head circumference, crown-heel length, weight, milk formula, and medications was recorded at each visit. Informed consent was obtained from the parents of all infants, and the study was approved by the St Joseph's Hospital Ethics Review Committee. In addition, 29 healthy adults receiving no medications were studied with the same laboratory methods as the infants.

Laboratory. Blood samples (2 mL) were usually obtained from a dorsal hand vein by using a 1½-in 21-gauge straight needle with the hub removed. The blood flowed directly into a premarked plastic tube containing 3.2% buffered sodium citrate (one part citrate to

nine parts blood). The samples were collected in the postnatal period on days 1, 5, 30, 90, and 180. The anticoagulant-to-blood ratio was not altered for the venous hematocrit because this value was not available. However, based on hematocrit values in the healthy newborn from our institution, 95% of the infants would have had an acceptable anticoagulant-to-blood-ratio.

The blood was immediately centrifuged (1,700 g) and platelet-poor plasma removed and frozen for future coagulation studies. Each 2 mL of blood gave a minimum of 600 μL of plasma, and all assays were performed by using previously published microtechniques.¹⁷ The coagulation screening tests, consisting of a prothrombin time (PT) (Dade C rabbit thromboplastin), activated partial thromboplastin time (APTT) (Dade FS), and 2-unit thrombin clotting time (TCT), used 20 μL each. Fibrinogen was measured as a thrombin clottable protein using 10 μL of plasma.¹⁸

The following coagulation factor assays were performed with human deficient plasma and 10 to 30 μL of each infant's plasma. Factors VIII-, IX-, XI-, and XII-deficient plasmas were obtained in-house from known severely deficient patients (<0.01 U/mL). High-molecular weight kininogen (HMW-K)- and prekallikrein (PK)-deficient plasmas were obtained from George King Biomedical, Overland Park, KS and factor VII-deficient plasma was

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Table 1. Demographic Data on the Study Population

	Postnatal Age (d)				
	1	5	30	90	180
Number	72	77	66	62	52
Weight* (kg)	3.50 ± 1.064	3.31 ± 1.062	4.54 ± 1.122	6.05 ± 1.596	7.62 ± 2.154
Head circumference* (cm)†	43.3 ± 2.3	34.2 ± 5.2	34.2 ± 5.4	37.4 ± 4.9	40.3 ± 3.2
Length* (cm)‡	50.6 ± 8.4	50.4 ± 8.9	53.4 ± 6.6	59.2 ± 6.4	64.8 ± 6.4
Breast-fed	70%	50%	41%	25%	15%

*Mean ± 1 SD.

†Maximal frontal occipital head circumference.

‡Crown-heel length.

prepared by immunodepletion of normal plasma (Interhaematol, Burlington, Ontario). Factor V-deficient plasma was prepared in-house by prolonged incubation of normal plasma collected into sodium oxalate. Factor II-deficient plasma was prepared from bovine plasma by adsorption with tricalcium phosphate. Factor X-deficient plasma was purchased from Sigma Chemical Co, St Louis, as VII-, X-, and bovine-deficient plasma. Factor XIII, subunits A and S, was measured by using Laurel immunoelectrophoresis. The antibodies were obtained commercially from Behring (Hoescht, Montreal). The plasma protease inhibitors antithrombin III (AT-III), α_2 -antiplasmin (α_2 -AP), C1 esterase inhibitor (C1E-INH), α_2 -macroglobulin (α_2 -M), and α_1 -antitrypsin (α_1 -AT) were all measured immunologically with commercially available rabbit antihuman antibody (Atlantic Antibodies, NCS Diagnostics, Inc, Mississauga, Ontario) by using a radial immunodiffusion that required 3 to 5 μ L per assay. Heparin cofactor II (HCII) was measured biologically on 50 μ L of plasma by a previously published method.¹⁹ Protein C was measured by an enzyme-linked immunosorbent assay (ELISA) (Diagnostica Stago, Wellmark Diagnostics, Guelph, Ontario). von Willebrand factor (vWF) was also measured by an ELISA method.²⁰ Protein S (Interhaematol) was measured by quantitative immunoelectrophoresis using rabbit antihuman protein S antibody.²¹ PK and plasminogen were each measured biologically on 10 to 20 μ L of plasma by chromogenic assays.^{22,23}

The sample size calculation was based on previously published

data. The α and β errors were set at 0.05 and 0.1, one-tailed, respectively. The change in time over the postnatal period for each factor level was measured by a one-way analysis of variance. The newborn values at each time were compared with the adult values to determine when they were statistically indistinguishable by using Dunnett's test. All *P* values < .05 were considered significant.

RESULTS

Subjects. Of 359 mothers interviewed, 118 agreed to have their infants join the study. Of these 118 mothers, 93 agreed to return for follow-up appointments. Between 61 and 77 infants returned and were studied at each time point for each assay. Not all infants were studied at each time point, primarily because of failure to return for follow-up, recruitment after day 1 of life, discharge before day 5, and occasionally clotted samples. The infant population was white (98%), with an equal proportion of males (53%) and females (47%). The mode of delivery was vaginal (98%), and all infants had a five-minute Apgar score ≥ 7 . The mean values for the weight, head circumference, and length of the infants in our study population at each time point are given in Table 1 as well as the proportion who were breast-fed at each time point.

Table 2. Reference Values for Coagulation Tests in the Healthy Full-term Infant During the First 6 Months of Life

Tests	Day 1 (n)	Day 5 (n)	Day 30 (n)	Day 90 (n)	Day 180 (n)	Adult (n)
PT (s)	13.0 ± 1.43 (61)*	12.4 ± 1.46 (77)*†	11.8 ± 1.25 (67)*†	11.9 ± 1.15 (62)*	12.3 ± 0.79 (47)*	12.4 ± 0.78 (29)
APTT (s)	42.9 ± 5.80 (61)	42.6 ± 8.62 (76)	40.4 ± 7.42 (67)	37.1 ± 6.52 (62)*	35.5 ± 3.71 (47)*	33.5 ± 3.44 (29)
TCT (s)	23.5 ± 2.38 (58)*	23.1 ± 3.07 (64)†	24.3 ± 2.44 (53)*	25.1 ± 2.32 (52)*	25.5 ± 2.86 (41)*	25.0 ± 2.66 (19)
Fibrinogen (g/L)	2.83 ± 0.58 (61)*	3.12 ± 0.75 (77)*	2.70 ± 0.54 (67)*	2.43 ± 0.68 (60)*†	2.51 ± 0.68 (47)*†	2.78 ± 0.61 (29)
II (U/mL)	0.48 ± 0.11 (61)	0.63 ± 0.15 (76)	0.68 ± 0.17 (67)	0.75 ± 0.15 (62)	0.88 ± 0.14 (47)	1.08 ± 0.19 (29)
V (U/mL)	0.72 ± 0.18 (61)	0.95 ± 0.25 (76)	0.98 ± 0.18 (67)	0.90 ± 0.21 (62)	0.91 ± 0.18 (47)	1.06 ± 0.22 (29)
VII (U/mL)	0.66 ± 0.19 (60)	0.89 ± 0.27 (75)	0.90 ± 0.24 (67)	0.91 ± 0.26 (62)	0.87 ± 0.20 (47)	1.05 ± 0.19 (29)
VIII (U/mL)	1.00 ± 0.39 (60)*†	0.88 ± 0.33 (75)*†	0.91 ± 0.33 (67)*†	0.79 ± 0.23 (62)*†	0.73 ± 0.18 (47)†	0.99 ± 0.25 (29)
vWF (U/mL)	1.53 ± 0.67 (40)†	1.40 ± 0.57 (43)†	1.28 ± 0.59 (40)†	1.18 ± 0.44 (40)†	1.07 ± 0.45 (46)†	0.92 ± 0.33 (29)†
IX (U/mL)	0.53 ± 0.19 (59)	0.53 ± 0.19 (75)	0.51 ± 0.15 (67)	0.67 ± 0.23 (62)	0.86 ± 0.25 (47)	1.09 ± 0.27 (29)
X (U/mL)	0.40 ± 0.14 (60)	0.49 ± 0.15 (76)	0.59 ± 0.14 (67)	0.71 ± 0.18 (62)	0.78 ± 0.20 (47)	1.06 ± 0.23 (29)
XI (U/mL)	0.38 ± 0.14 (60)	0.55 ± 0.16 (74)	0.53 ± 0.13 (67)	0.69 ± 0.14 (62)	0.86 ± 0.24 (47)	0.97 ± 0.15 (29)
XII (U/mL)	0.53 ± 0.20 (60)	0.47 ± 0.18 (75)	0.49 ± 0.16 (67)	0.67 ± 0.21 (62)	0.77 ± 0.19 (47)	1.08 ± 0.28 (29)
PK (U/mL)	0.37 ± 0.16 (45)†	0.48 ± 0.14 (51)	0.57 ± 0.17 (48)	0.73 ± 0.16 (46)	0.86 ± 0.15 (43)	1.12 ± 0.25 (29)
HMW-K (U/mL)	0.54 ± 0.24 (47)	0.74 ± 0.28 (63)	0.77 ± 0.22 (50)*	0.82 ± 0.32 (46)*	0.82 ± 0.23 (48)*	0.92 ± 0.22 (29)
XIIIa (U/mL)	0.79 ± 0.26 (44)	0.94 ± 0.25 (49)*	0.93 ± 0.27 (44)*	1.04 ± 0.34 (44)*	1.04 ± 0.29 (41)*	1.05 ± 0.25 (29)
XIIIb (U/mL)	0.76 ± 0.23 (44)	1.06 ± 0.37 (47)*	1.11 ± 0.36 (45)*	1.16 ± 0.34 (44)*	1.10 ± 0.30 (41)*	0.97 ± 0.20 (29)
Plasminogen (CTA, U/mL)	1.95 ± 0.35 (44)	2.17 ± 0.38 (60)	1.98 ± 0.36 (52)	2.48 ± 0.37 (44)	3.01 ± 0.40 (47)	3.36 ± 0.44 (29)

NOTE. All factors except fibrinogen and plasminogen are expressed as units per milliliter where pooled plasma contains 1.0 U/mL. Plasminogen units are those recommended by the Committee on Thrombolytic Agents (CTA). All values are expressed as mean ± 1 SD.

Abbreviation: VIII, factor VIII procoagulant.

*Values that do not differ statistically from the adult values.

†These measurements are skewed because of a disproportionate number of high values. The lower limit that excludes the lower 2.5th percentile of the population has been given in the respective figures. The lower limit for factor VIII was 0.50 U/mL at all time points for the infant.

Laboratory. Tables 2 and 3 give the mean ± 1 SD and the number of samples tested for the coagulation factors and inhibitors, respectively. Figures 1 to 7 give the mean values $\pm 95\%$ confidence intervals for the mean value and the upper and lower limits that encompass 95% of the population (± 2 SD) for each of the tests performed. The 95% confidence interval for the mean value of each test at each time point was very small (less than $\pm 0.06\%$) in the coagulation tests in this population, which allowed us to accurately detect changes over time. Values that were skewed were identified in Tables 2 and 3 and the lower limit adjusted to exclude 2.5% of the population. The values that do not differ significantly from those of the adult are identified in Tables 2 and 3.

Table 2 and Fig 1 give the values for the coagulation screening tests in the first 6 months of life. The PT significantly shortened during the first month of life but was always comparable to the adult value. The APTT, which was prolonged at birth, reached adult values by 3 months of age. The TCT was in the adult range from time of birth, as was the fibrinogen level; however, there was a significant rise in the fibrinogen level on day 5 of life.

Table 2 and Fig 2 give the values for the vitamin K-dependent factors (II, VII, IX, and X) in the first 6 months of life. Each of the vitamin K-dependent factors had a distinct postnatal pattern of maturation. Levels of factor VII rapidly rose to near adult levels by day 5 of life, which contributed to the shortening of the PT at this same postnatal time point. Levels of factors II, IX, and X showed a relatively delayed rise. All four vitamin K-dependent factors were in the adult range at 6 months of life; however, the mean values for all four factors were still significantly lower in the infant compared with the adult. There was no difference between breast-fed and formula-fed infants for any of the vitamin K-dependent factors at any time point.

Table 2 and Fig 3 give the values for the four contact factors (XII, XI, PK, and HMW-K) during the first 6 months of life. The levels of these factors were all low at the time of birth. Levels of HMW-K rapidly rose to adult values by 1 month of life, whereas levels of factors XI, XII, and PK showed a gradual rise into the adult range by 6 months of age. The mean values for factors XI, XII, and PK were still significantly lower in the infant compared with the adult at 6 months of age.

Table 2 and Figs 4 and 5 give the postnatal course for factors V, VIII, vWF, plasminogen, and factor XIII with its subcomponents A and B. The mean value for factor V was in the adult range at birth and rose to 0.95 U/mL by day 5 of

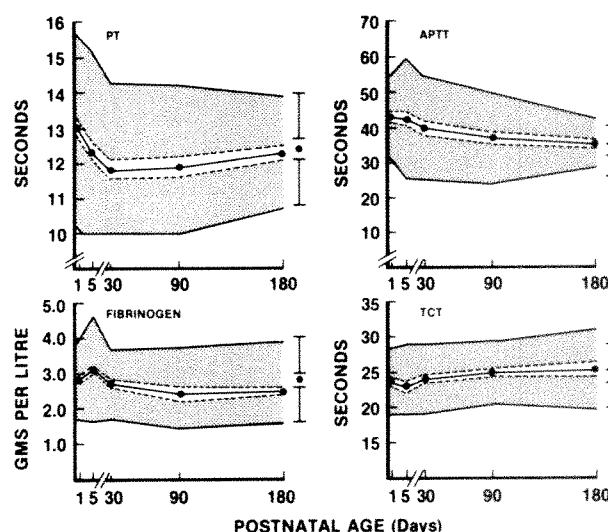


Fig 1. APTT, PT, fibrinogen level, and TCT in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD).

Adult values are indicated

life. Factor VIII and vWF showed a persistently skewed distribution with a number of very elevated values. The lower limit of normal has been adjusted to reflect the true limit excluding 2.5% of the population. The mean value for factor VIII at birth was the same as the adult, whereas levels of vWF were elevated compared with the adult. Levels for both factors VIII and vWF gradually decreased over the first 6 months of life. For factor VIII, only three values out of 306 tests were below 0.40 U/mL, and these three values were all above 0.30 U/mL. Factors XIIIa and XIIIb showed essentially identical patterns with indistinguishable mean values. Adult levels for both factors XIIIa and XIIIb were achieved by day 5 of life. Plasminogen values were approximately two thirds of adult values at birth and gradually rose to near-adult values by 6 months.

Table 3 and Fig 6 give the postnatal course for the inhibitors AT-III, HCII, protein C, and protein S. All of these inhibitors had low levels at the time of birth. The mean values for AT-III were similar for the infant and adult by 3 months of age, whereas the mean value for HCII was high in the infant at 6 months of age compared with the adult. Protein C still had markedly low levels at 6 months of age.

Table 3 and Fig 7 give the values for the inhibitors, α_2 -M,

Table 3. Reference Values for the Inhibition of Coagulation in the Healthy Full-term Infant During the First 6 Months of Life

Inhibitors	Day 1 (n)	Day 5 (n)	Day 30 (n)	Day 90 (n)	Day 180 (n)	Adult (n)
AT-III	0.63 $\pm$ 0.12 (58)	0.67 $\pm$ 0.13 (74)	0.78 $\pm$ 0.15 (66)	0.97 $\pm$ 0.12 (60)*	1.04 $\pm$ 0.10 (56)*	1.05 $\pm$ 0.13 (28)
α_2 -M	1.39 $\pm$ 0.22 (54)	1.48 $\pm$ 0.25 (73)	1.50 $\pm$ 0.22 (61)	1.76 $\pm$ 0.25 (55)	1.91 $\pm$ 0.21 (55)	0.86 $\pm$ 0.17 (29)
α_2 -AP	0.85 $\pm$ 0.15 (55)	1.00 $\pm$ 0.15 (75)*	1.00 $\pm$ 0.12 (62)*	1.08 $\pm$ 0.16 (55)*	1.11 $\pm$ 0.14 (53)*	1.02 $\pm$ 0.17 (29)
C.E-INH	0.72 $\pm$ 0.18 (59)	0.90 $\pm$ 0.15 (76)*	0.89 $\pm$ 0.21 (63)	1.15 $\pm$ 0.22 (55)	1.41 $\pm$ 0.26 (55)	1.01 $\pm$ 0.15 (29)
α_1 -AT	0.93 $\pm$ 0.22 (57)*	0.89 $\pm$ 0.20 (75)*	0.62 $\pm$ 0.13 (61)	0.72 $\pm$ 0.15 (56)	0.77 $\pm$ 0.15 (55)	0.93 $\pm$ 0.19 (29)
HCII	0.43 $\pm$ 0.25 (56)	0.48 $\pm$ 0.24 (72)	0.47 $\pm$ 0.20 (58)	0.72 $\pm$ 0.37 (58)	1.20 $\pm$ 0.35 (55)	0.96 $\pm$ 0.15 (29)
Protein C	0.35 $\pm$ 0.09 (41)	0.42 $\pm$ 0.11 (44)	0.43 $\pm$ 0.11 (43)	0.54 $\pm$ 0.13 (44)	0.59 $\pm$ 0.11 (52)	0.96 $\pm$ 0.16 (28)
Protein S	0.36 $\pm$ 0.12 (40)	0.50 $\pm$ 0.14 (48)	0.63 $\pm$ 0.15 (41)	0.86 $\pm$ 0.16 (46)*	0.87 $\pm$ 0.16 (49)*	0.92 $\pm$ 0.16 (29)

NOTE. All values are expressed in units per milliliter as the mean ± 1 SD.

*Values that do not differ statistically from the adult values.

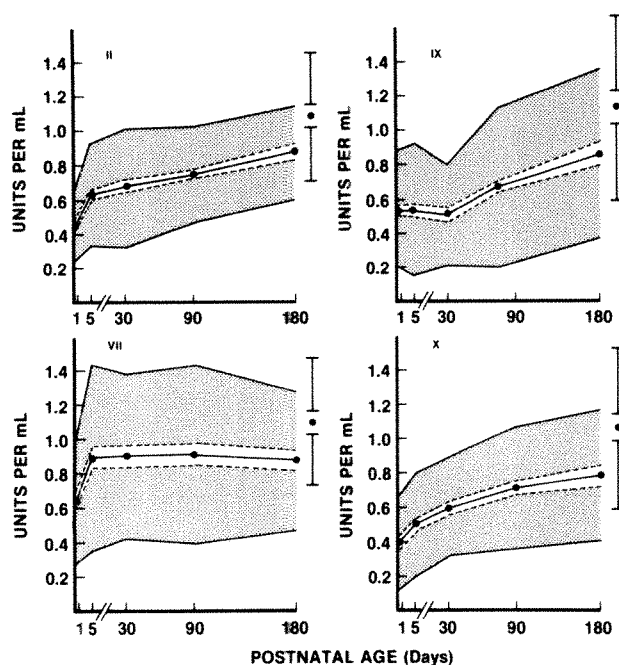


Fig 2. The vitamin K-dependent factors (II, VII, IX, X) in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD). Adult values are indicated

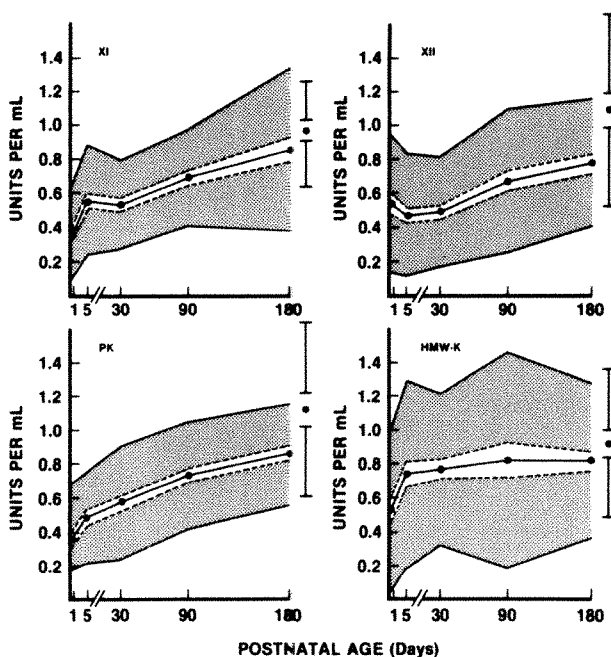


Fig 3. The contact factors (XII, XI, PK, HMW-K) in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD).

Adult values are indicated

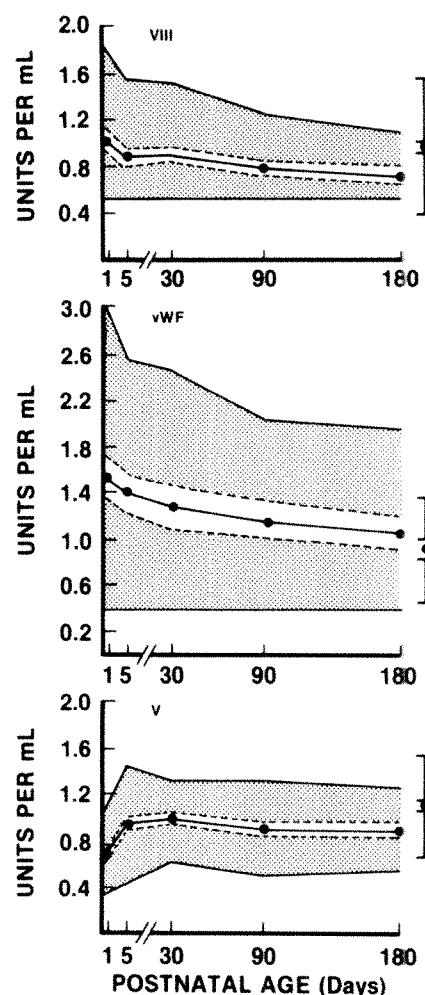


Fig 4. Factors V, VIII, and vWF in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD). Adult values are indicated

C₁E-INH, α_2 -AP and α_1 -AT. Levels for α_2 -M were elevated at birth compared with the adult, and these values continued to rise in the postnatal period. Although levels for C₁E-INH were low at birth compared with the adult, the levels for C₁E-INH also continued to rise well above adult values during the first 6 months of life. In contrast, levels of α_2 -AP at birth were in the lower part of the adult range and achieved the adult values by day 5 of life. The levels of α_2 -AP then remained constant over the first 6 months of life. Levels of α_1 -AT were similar in the neonate compared with the adult; however, the levels of α_1 -AT in the neonate fell by 3 months of age and by 6 months of age had begun to increase again.

DISCUSSION

A knowledge of the normal postnatal development of the human coagulation system is essential to investigate thrombotic and hemorrhagic problems in the neonate. The definition of normal values of any one parameter in a single population is a difficult task. Physicians caring for infants

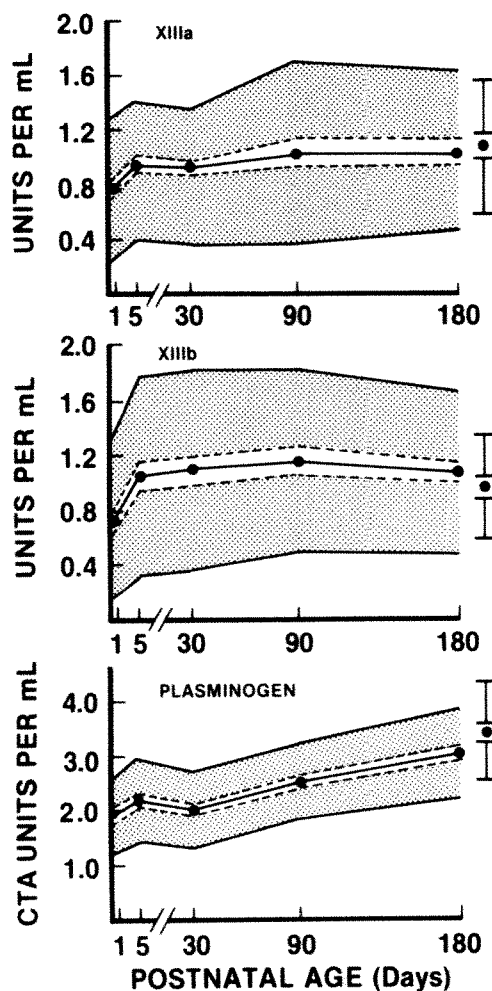


Fig 5. Factor XIIIa, XIIIb, and plasminogen in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD). Adult values are indicated

with hemostatic problems are faced with a need to establish normal values for at least 25 coagulation parameters that are known to be constantly changing over the first few weeks to months of life. To complicate matters further, these changing parameters are not only dependent upon the postnatal age of the infant but are also dependent upon the gestational age. The problem is further compounded by the difficulty of obtaining adequate blood samples, the small size of the sample that can be taken, the necessity of using microtechniques to perform the multiple assays on small samples, and the large number of infants who must be studied because of the greater variability that exists at any single time point for any coagulation measurement in the neonatal population compared with the adult. Because of these difficulties, the normal postnatal development of the human coagulation system in the full-term infant is still largely unknown. Our study was designed to determine the normal postnatal values for the components of the coagulation, fibrinolytic, and inhibitor systems in the healthy full-term infant.

The PT, APTT, TCT, and fibrinogen levels are the most

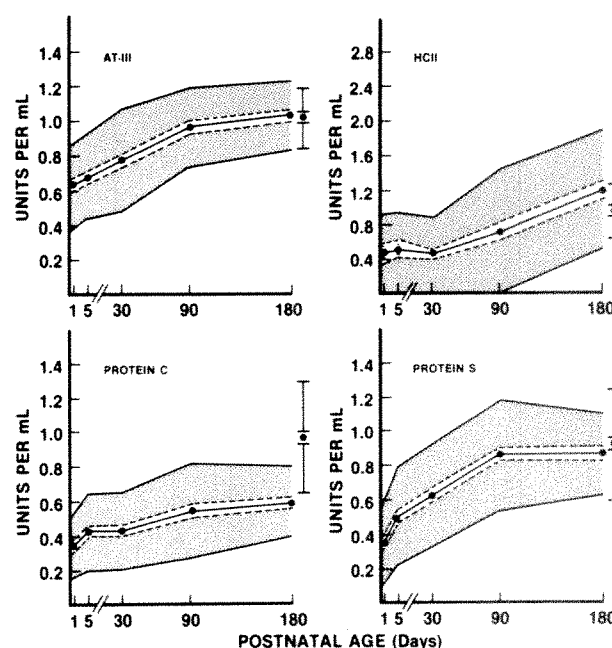


Fig 6. The inhibitors AT-III, HCII, protein C, and protein S in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD). Adult values are indicated

commonly used screening tests in infants with a suspected coagulopathy. There are reference mean values published for these tests in the first week of life¹⁻¹⁶; however, this study has extended our information for these tests in a number of ways. Previously the mean PT values have been reported to be prolonged at birth and to reach adult values by a week of life. This study showed that although the variability of the PT was greater in the newborn, the mean values were not significantly different from the adult. The PT did shorten in the newborn during the first month of life. Previously reported values for the APTT in the neonate were so prolonged that the usefulness of the APTT as a diagnostic test in the infant was questionable.^{1,4} By using a reagent containing ellagic acid rather than kaolin as an activating agent we were able to considerably narrow the ranges of normal, which may increase the usefulness of the APTT as a screening test in the neonate. In the neonate, the TCT can be of limited value when performed without calcium in the buffering system because under these conditions the TCT may be prolonged due to a normally present fetal fibrinogen.²⁴ In this study, the TCT was measured with calcium present in the buffering system so that a narrow normal range was delineated and abnormal values secondary to the presence of heparin or low levels of fibrinogen could be easily detected. Previously, an elevated fibrinogen level has been used as an indicator of sepsis in the neonate.^{25,26} The normally elevated levels of fibrinogen on day 5 of life suggests that this is at least in part a physiological postnatal phenomenon and may not be indicative of sepsis.

The levels of the four vitamin K-dependent factors have been extensively studied in the full-term infant.²⁷⁻³² Vitamin

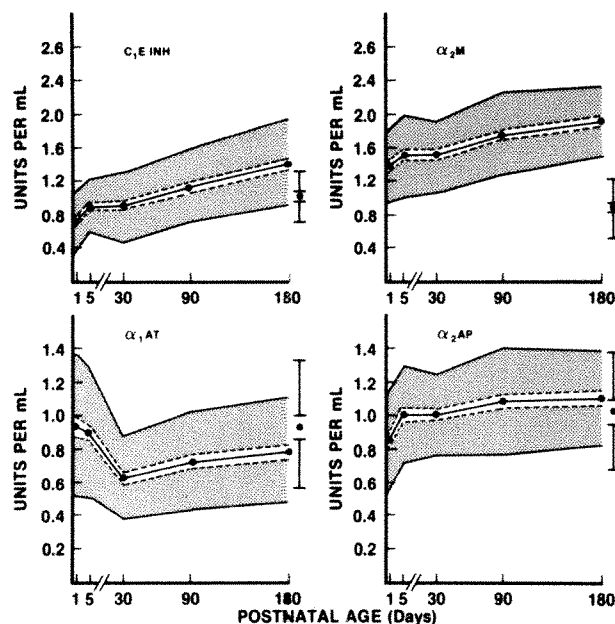


Fig 7. The inhibitors C₁E-INH, α_2 -M, α_2 -AP, and α_1 -AT in 118 healthy full-term infants throughout the first 6 months of life. The inner line represents the mean values, the inner clear area the 95% confidence interval, and the shaded area 95% of all values (± 2 SD). Adult values are indicated

K deficiency can occur in the full-term neonate although the true incidence and frequency of bleeding manifestations is unclear.³³ All of our infants received vitamin K at birth, so our reference values may apply only to other infants who have received vitamin K at the time of birth. Vitamin K deficiency has also been reported to occur later in the postnatal period in infants who have received vitamin K at birth and are breast-fed.²⁹ Our sample size was too small to determine with accuracy the frequency of vitamin K deficiency at 1 month in infants who received vitamin K at birth. However, we subanalyzed our data to compare breast-fed and formula-fed infants for all of the vitamin K-dependent factors at each time point, and there was no significant difference detected, which is compatible with previously reported data at 1 month of age.²⁹ Finally, our data showed that the severe form of Christmas disease or factor IX deficiency can be diagnosed at the time of birth; however, moderate and mild forms are likely not reliably diagnosed.

The levels for the four contact factors (XII, XI, PK, HWM-K) were low at birth and gradually increased to values approaching the adult values by 6 months of life. The low levels of these four factors likely contribute to the prolonged APTT during the first 6 months of life.^{4,34-39} The

homozygous form of factor XI can be reliably detected as early as day 5 of life because the lower limit of our range at this time was 0.20 U/mL, which is above the levels for homozygous factor XI-deficient patients.

The factor VIII molecule has been extensively studied in the immediate postnatal period.⁴⁰⁻⁴⁷ Our study showed similar results; however, the values for factor VIII were skewed, with many infants having elevated values at the time of birth. Less than 1% of the values for factor VIII were below 0.40 U/mL, and all values were above 0.30 U/mL. Thus, severe and moderate hemophilia A can be reliably diagnosed in the immediate postnatal period. vWF levels have been reported to be elevated in healthy full-term infants, and our data would be compatible with the published results.^{40,42-45,47} In addition, our study showed that vWF levels remain elevated until 3 months of age, thus suggesting that the elevated levels were not a brief response to the birth process itself.

Some of the inhibitors of the coagulation system have been studied in part.⁴⁸⁻⁵⁸ In our study, levels of C₁E-INH, α_2 -M, and HCII became elevated during the first 6 months of life compared with the adult. The significance of these elevated values is unknown, but one may speculate that this may be in part compensation for the low levels of other inhibitors. Levels of AT-III, protein C, protein S, and HCII were low in the first weeks of life and all in the range in which thrombotic disorders have been described in the adult. Of some interest, protein C still had markedly depressed levels at 6 months of age. All of the inhibitors in our study except for HCII were measured by immunologic assays. Whether any of these proteins have lower biologic activity compared with immunologic levels has been questioned by other investigators but was not addressed in this particular study.^{49,58}

In summary, our study has determined the postnatal development of the human coagulation system in a large number of consecutively studied healthy full-term infants. Utilizing these reference values, future studies will be directed at the determination of the epidemiology and clinical impact of specific abnormalities and the role of therapeutic interventions in specific hemorrhagic and thrombotic problems in the neonate.

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Proteolytic Degradation of von Willebrand Factor After DDAVP Administration in Normal Individuals

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The infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) in normal individuals is followed by an increase in factor VIII/von Willebrand factor (vWF) in plasma, by an increase in intensity of all sizes of multimers, and by the appearance of larger multimers of vWF than those seen in the resting state. Since the larger multimers are rapidly cleared and proteolysis is known to cause disaggregation of large multimers, we evaluated the degree of vWF proteolysis after DDAVP administration. DDAVP was infused into eight normal adult volunteers, and the relative proportions of the intact 225 kilodalton (kDa) subunit and the 189, 176, and 140 kDa vWF fragments were compared before and at different times after DDAVP infusion. The

relative proportion of the 176 kDa fragment was increased, whereas that of the other species was decreased, thereby indicating that proteolytic fragmentation had occurred. However, plasmin did not appear to be responsible because the vWF fragments characteristically produced by this enzyme could not be detected. Concomitant analysis of vWF multimeric structure showed that these changes were accompanied by an increase in the relative proportion of the satellite bands, which suggests that they were proteolytically generated. Proteolysis may explain, at least in part, rapid clearance of larger vWF multimers released by DDAVP.

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RECENTLY it has been demonstrated that a small but consistent proportion of the von Willebrand factor (vWF) in normal plasma is composed of fragments with apparent molecular masses of 189, 176, and 140 kilodaltons (kDa) that have been cleaved from the 225 kDa subunit, indicating that proteolysis normally plays a role in the processing of vWF.¹

The infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) in normal individuals is followed by an increase in vWF levels, by an increase in the intensity of all sizes of multimers, and by the appearance of multimers that are larger than those seen in the resting state and that are progressively cleared.² Also, DDAVP releases plasminogen activator.^{3,4}

To better understand the processing of this DDAVP-released vWF we have studied the vWF multimeric and subunit composition in normal individuals at baseline and after DDAVP infusion. We have found that at least to some extent DDAVP infusion is accompanied by the induction of proteolytic degradation of vWF. Monoclonal antibody mapping of the fragments indicated that plasmin was not responsible. Thus, the increased fragmentation could not have resulted from plasminogen activation subsequent to plasminogen activator release by DDAVP. However, the enzyme or enzymes responsible are unknown at present.

Previous studies have provided evidence that the vWF molecule in IIA von Willebrand's disease (vWD) is more susceptible to cleavage than normal vWF.^{1,5} The results presented here suggest that the failure of DDAVP to control bleeding in some individuals with IIA vWD may result from an unfavorable balance between release of large multimers and their proteolytic degradation.

MATERIALS AND METHODS

Reagents. Leupeptin and *N*-ethylmaleimide (NEM) were acquired from Sigma Chemical Co, St Louis; electrophoresis pure reagents from Bio-Rad, Richmond, CA; and rabbit antimouse IgG was from Zymed, South San Francisco. All other reagents were of the highest grade available.

Patients. Eight normal volunteers, five males and three females aged between 18 and 36 years with no drug intake for at least 20 days before the study, were included. DDAVP (Minirin, Ferring

Laboratories, Malmo, Sweden) at a dose of 0.4 µg/kg body weight was diluted with 100 mL of normal saline and infused intravenously over a 30-minute period. vWF antigen (vWF:Ag) and ristocetin cofactor activity were determined as described.⁶ Blood was drawn before (0) and after (30 and 60 minutes and 2, 4, and 24 hours, respectively) DDAVP administration. Plasma samples were obtained in the presence of 1 mmol/L leupeptin, 6 mmol/L NEM, and 5 mmol/L EDTA as described.⁶

Immunoisolation of vWF. vWF was immunisolated by incubation of 1 to 5 mL of plasma with 1 to 2.5 mL of agarose beads covalently linked to anti-vWF monoclonal antibody as described.¹ After extensive washing the vWF was eluted with 2 mL of 2% sodium dodecyl sulfate (SDS) and 0.1 mol/L Tris (pH 8.0) at 60°C, and the eluate was concentrated by using Amicon Centricron 30 microconcentrators (Amicon Corp, Danvers, MA) to a final volume of approximately 100 µL.¹

SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-5% polyacrylamide gels were prepared according to Laemmli.⁷ The vWF was reduced by dithiothreitol (final concentration, 65 mmol/L) for 15 minutes at 60°C in pH 7.0 polyacrylamide gel sample buffer. After SDS-5% polyacrylamide electrophoresis the samples were electrophoretically transferred to nitrocellulose

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and then reacted with a pool of 55 anti-vWF monoclonal antibodies, all of which reacted with the reduced 225 kDa subunit.¹ The nitrocellulose was then reacted with ¹²⁵I-rabbit antimouse IgG antibody. The relative concentrations of the intact vWF subunit and its proteolytic fragments were determined by identifying each band by autoradiography and then excising each band and counting them in a γ -scintillation counter (Clinigamma, LKB, Bromma, Sweden).

Immunoblots were also made with monoclonal antibody pools that reacted only with an amino-terminal cyanogen bromide fragment (M7) or a carboxy-terminal cyanogen bromide fragment (M31) and that differentiated between the "native" 176 and 140 kDa fragments and the plasmin-cleaved fragments of similar size.⁸

SDS-agarose electrophoresis. For analysis of vWF multimers this procedure was performed as described.⁹ In brief, thin-layer agarose electrophoresis was performed in the presence of SDS by using a discontinuous buffer system. vWF multimers were identified autoradiographically by reacting the gels with [¹²⁵I]-labeled affinity purified anti-vWF rabbit antibody.¹⁰

Statistical analyses. To analyze the evolution of the proteolytic fragments of vWF induced by the infusion of DDAVP, we first studied the mean percentage of counts per minute of each polypeptide at six different times (0, 30, and 60 minutes and 2, 4, and 24 hours) in the eight individuals. We corroborated the normality of the distributions by using the STAWORKS program. The mean values of each band at different times were then compared with each other by both a parametric test (Student's *t* test) and a nonparametric test (Wilcoxon signed rank), and the *P* value was calculated. As a consequence of the need to make multiple comparisons of variable pairs, which can artifactually increase the level of statistical significance, we calculated the maximum probability of error admissible by using the Bonferroni method, a procedure that determines the acceptable maximal error to be admitted. In that regard a maximal error of 5% was admitted. In addition, we have analyzed the evolution of each band in comparison with the others by using the Spearman correlation test to determine whether they are dependent on or independent of each other's evolution.¹¹

RESULTS

DDAVP infusion produced a concomitant increase in vWF:Ag and ristocetin cofactor activity to about two or three times the resting concentration, being maximal at 30 to 120 minutes, as already described.² All multimers of vWF showed an increase in plasma concentration, and larger multimers than those initially present in plasma also appeared and reached a maximum at 30 minutes (Fig 1). The larger multimers disappeared from the circulation by two to four hours. With higher-resolution gels, an increase in the relative proportion of the satellite bands, as compared with the predominant central band, of each repeating multimeric unit was observed. This alteration was maximal approximately 60 minutes after the infusion and was almost entirely gone by 24 hours (Fig 2).

Immunoblots made with the pool of 55 anti-vWF monoclonal antibodies showed a significant increase in the relative concentration of the 176 kDa fragment in comparison with that of the intact 225 kDa subunit and the 189 and 140 kDa fragments. This change was already present at 30 minutes (*P* < .006) and became maximal at two hours after the infusion (Figs 3 and 4). At 24 hours the subunit pattern was similar to that observed in the resting state.

To evaluate the possibility that the increase in the 176 kDa fragment represented an increase in the species normally

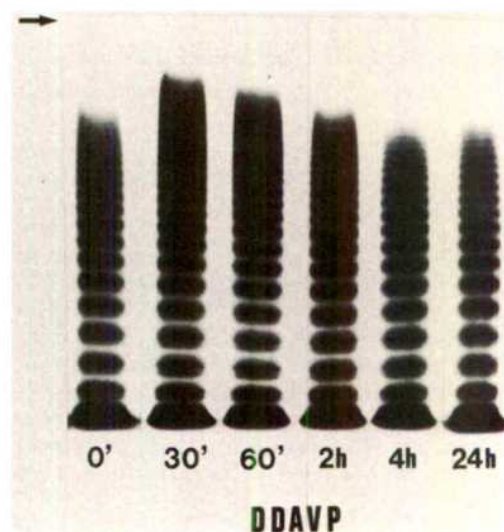


Fig 1. Autoradiograph of SDS-1% agarose gel electrophoresis of normal plasma before (0) and after (30 and 60 minutes and 2, 4, and 24 hours, respectively) the administration of DDAVP. Multimers of vWF larger than those seen in the basal state appear by 30 minutes after the infusion, with a progressive disappearance and a return to the resting pattern at 24 hours. The arrow marks the interface between the stacking (sample) and running gels.

present in plasma or resulted from plasmin cleavage consequent to the release of plasminogen activator by DDAVP, monoclonal antibody mapping studies were performed. A pool of monoclonal antibodies reactive with a cyanogen bromide fragment derived from the amino-terminal portion

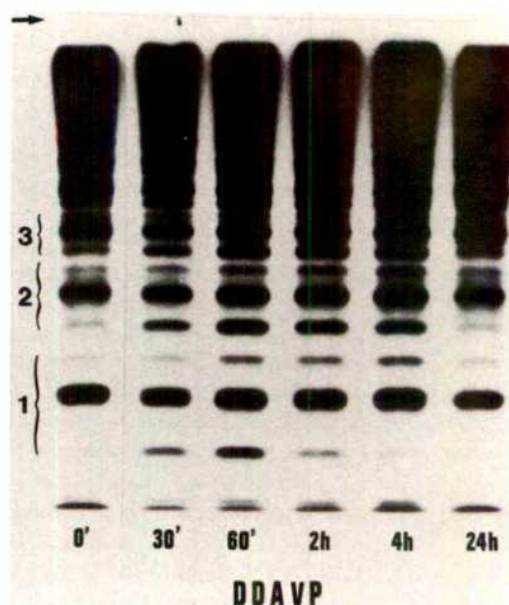


Fig 2. Autoradiograph of SDS-1.6% agarose gel electrophoresis of normal plasma at the same times as in Fig 1. A transient increase in the relative proportion of the satellite bands, as compared with the central predominant band, of the smallest two multimers (brackets) became evident after DDAVP administration. The arrow marks the interface between the stacking (sample) and running gels.

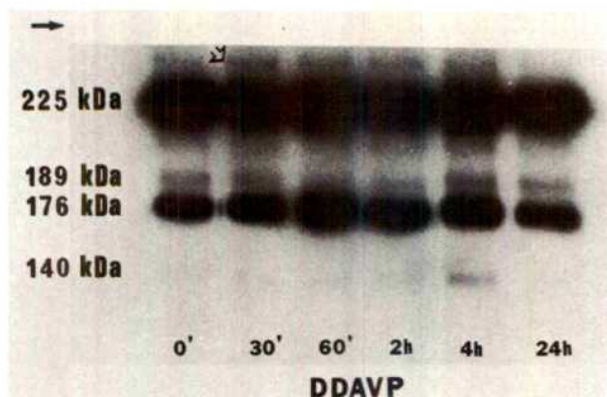


Fig 3. Immunoblot autoradiograph of reduced immunoprecipitated vWF before (0) and after (30 and 60 minutes and 2, 4, and 24 hours, respectively) the infusion of DDAVP. vWF was reacted with a pool of 55 anti-vWF monoclonal antibodies. An increase of the 176 kDa fragment relative to that of the 225, 189, and 140 kDa species was evident after DDAVP administration; recovery occurred by 24 hours. A band migrating more slowly than the 225 kDa subunit is partially reduced vWF (white arrow). The exposure time of this autoradiograph was too short to allow good visualization of the 140 kDa fragment. The black arrow marks the interface between the stacking (sample) and running gels.

of the 2,050-amino acid residue vWF subunit (M7, residues 185 to 288)¹² and a pool of monoclonal antibodies reactive with a cyanogen bromide fragment from the carboxy-terminal portion of the subunit (M31, residues 1,481 to 1,693)¹² were used in the immunoblots in place of the 55-antibody pool.

The M7 antibodies react with the 140 kDa fragment present in normal plasma in the resting state, which is therefore derived from the amino-terminal portion of the molecule, and with a 176 kDa plasmin-derived amino-terminal fragment.⁸ This pool of antibodies reacted only with the 140 kDa species in the post-DDAVP specimens, thereby indicating that the increased quantity of the 176 kDa species

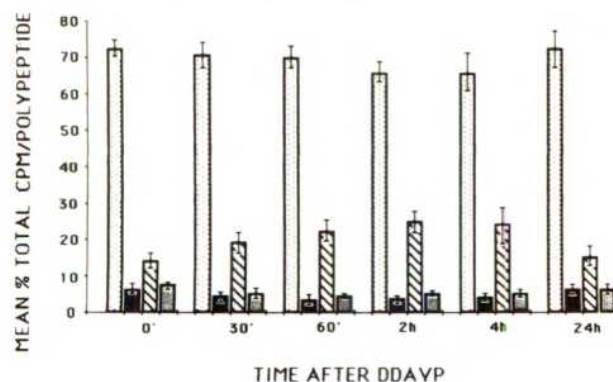


Fig 4. Relative proportion of counts in each of the bands shown in Fig 3 as determined by excising each band from nitrocellulose and measuring γ -scintillation counts (mean percentage of total counts per minute of each polypeptide). Brackets indicate ± 1 SD. An increase in the 176 kDa polypeptide relative to the other species was evident after DDAVP administration; return to normal occurred by 24 hours. $\square$, 225 kDa; $\blacksquare$, 189 kDa; $\square$, 176 kDa; $\blacksquare$, 140 kDa.

was not attributable to a plasmin-cleaved product of this size (Fig 5).

The M31 antibodies, on the other hand, react with the 176 kDa fragment present in normal plasma in the resting state, which is therefore carboxy-terminal in its derivation, and with a 145 kDa carboxy-terminal plasmin cleavage product.⁸ These antibodies detected only a native 176-kDa species in the post-DDAVP specimens, again providing evidence that plasmin cleavage of vWF had not occurred (not shown).

DISCUSSION

In vivo proteolysis of vWF has recently been demonstrated in normal individuals by showing that a small but consistent proportion of the vWF 225 kDa subunit is present in normal plasma as cleaved fragments with apparent molecular masses of 189, 176, and 140 kDa.¹ In type IIA vWD the relative proportion of the 176 and 140 kDa fragments is increased, which supports the hypothesis that proteolysis is responsible, at least in part, for the absence of large multimers in that disorder.^{1,5,6} Plasmin, the platelet calcium-activated protease (calpain), and one or more enzymes released from polymorphonuclear leukocytes proteolyze vWF in vitro, with resultant loss of large multimers.^{6,13-16} However, it is not clear whether any of these enzymes has a similar effect in vivo.

By using monoclonal antibodies to map the fragments to different portions of the vWF subunit it was previously

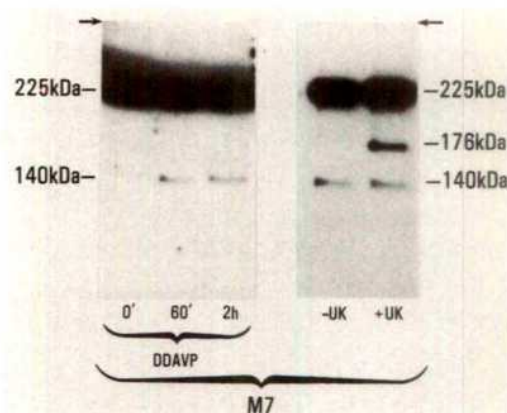


Fig 5. Immunoblot autoradiograph of reduced immunoprecipitated vWF reacted with M7 monoclonal antibodies. Left panel: normal plasma vWF before (0) and after (60 minutes and two hours, respectively) DDAVP administration and drawn in the presence of 1 mmol/L leupeptin, 6 mmol/L NEM, and 5 mmol/L EDTA. Right panel: vWF from normal untreated plasma (-UK) and vWF from plasma reacted with urokinase (+UK). Normal untreated plasma was drawn in citrate anticoagulant with the three proteinase inhibitors as before. Plasma to be reacted with urokinase (384 Ploug U/mL of plasma) was drawn in citrate anticoagulant alone and incubated with the enzyme at 37°C for 16 hours.⁸ The 176 kDa amino-terminal fragment produced by plasmin is present in the in vitro urokinase-treated (+UK) sample only (right panel) and could not be detected in vivo before or after DDAVP administration (left panel). The amino-terminal native 140 kDa fragment is present in plasma before and after UK treatment and is slightly more prominent after DDAVP infusion. The arrow marks the interface between the stacking (sample) and running gels.

shown that the increased proportion of fragments in IIA vWD was not a result of plasmin proteolysis.⁸ Rather, the fragments reacted with the same monoclonal antibodies that the 140 and 176 kDa fragments reacted with in normal plasma, thereby indicating that the IIA fragments resulted from a degradative process analogous to that operative in normal individuals. Similarly, the post-DDAVP increase in the 176 kDa fragment reported here was not the result of plasmin cleavage, even though plasminogen activator is released by DDAVP. Thus, the enzyme or enzymes responsible for the increased cleavage vWF in normal individuals, or in IIA vWD, is uncertain at present.

The infusion of DDAVP in normal individuals was followed by the appearance of multimers that were larger than those seen in the resting state and that disappeared over time (Fig 1). Using higher-resolution gels we observed that after DDAVP infusion the structure of each multimer changed; it showed an increase in the satellite bands (Fig 2). This increase in satellite bands is reminiscent of that seen in type IIA vWD,^{1,6} suggesting that in both situations they are the result of increased proteolysis.

After DDAVP administration, increased proportions of von Willebrand fragments circulate, but the mechanism by which this occurs is uncertain at present. DDAVP causes the release of larger, presumably more hemostatically effective, multimers from storage sites and also increases the overall concentration of vWF in plasma. It is possible that these larger multimers are more susceptible to normal degradative processes and thus are rapidly fragmented. Alternatively, DDAVP may release one or more enzymes or enzyme activators and in this manner accelerate the loss of large multimers. In either case, identifying the enzyme or enzymes responsible should allow the development of strategies for making DDAVP more effective, particularly in IIA vWD where the vWF appears to be more susceptible to cleavage.^{2,5}

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Regulation of Thrombopoiesis: Effects of the Degree of Thrombocytopenia on Megakaryocyte Ploidy and Platelet Volume

By Laurence Corash, Huei Yin Chen, Jack Levin, Georgiann Baker, Helen Lu, and Yuen Mok

We have established a murine model and techniques with which to serially study thrombopoiesis after induction of experimental immune thrombocytopenia of variable severity and duration. Bone marrow megakaryocyte ploidy distribution was determined by using unfractionated bone marrow, a polyclonal megakaryocyte-specific probe, and two-color, fluorescence-activated flow cytometry. With these techniques, the modal megakaryocyte ploidy class in normal murine bone marrow was 16N. Serial studies of bone marrow megakaryocyte ploidy after the induction of acute, severe thrombocytopenia (platelet count, $<0.05 \times 10^6/\mu\text{L}$) demonstrated no detectable change in the ploidy distribution at 12, 24, and 36 hours after the onset of thrombocytopenia. At 48 hours, the modal ploidy class shifted from 16N to 32N, and the 64N class increased significantly ($P < .001$). The ploidy distribution returned to normal 120 hours after the onset of thrombocytopenia. A lesser degree of thrombocytopenia (platelet count reduction to 0.100 to $0.200 \times 10^6/\mu\text{L}$) delayed the modal ploidy class shift from 16N to 32N until 72 hours after the onset of thrombocytopenia. Chronic, severe thrombocytopenia

(platelet count, $<0.05 \times 10^6/\mu\text{L}$ for seven days) resulted in a modal ploidy class shift from 16N to 32N during the thrombocytopenic phase and an enhanced increase in the 64N megakaryocyte class during the recovery phase. Mean platelet volume (MPV) was simultaneously measured on isolated total platelet populations after induction of thrombocytopenia. MPV was significantly increased ($P < .001$) as early as eight hours after the onset of acute, severe thrombocytopenia, 40 hours before a shift in the ploidy distribution. Mild thrombocytopenia (platelet count reduction to $0.400 \times 10^6/\mu\text{L}$) was not associated with a ploidy shift but did result in a significantly increased MPV ($P < .001$). These studies demonstrate that the temporal relationship and magnitude of the effects of thrombocytopenia upon megakaryocyte ploidy distribution are dependent upon the degree and the duration of the thrombocytopenic stimulus and that the effects of experimental thrombocytopenia on platelet volume and megakaryocyte ploidy are dissociated.

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THE MECHANISMS that regulate thrombopoiesis remain incompletely understood. Previous studies suggest that experimental, acute thrombocytopenia results in alterations of platelet volume,^{1,2} megakaryocyte cytoplasmic volume,³⁻⁷ stages of megakaryocyte maturation,^{1,3,4} megakaryocyte ploidy distribution,^{1,4,6,8,9} and megakaryocyte colony-forming cells (Meg-CFC).¹⁰⁻³¹ While these studies indicate that thrombopoiesis is responsive to fluctuations in the circulating platelet mass, considerable controversy persists concerning many aspects of thrombopoiesis, including the normal megakaryocyte ploidy distribution,^{9,14,15} the determinants of platelet volume,^{16,17} and the response of Meg-CFC to thrombocytopenia.^{18,19} A majority of studies of thrombopoietic regulation^{1,3,4,6,7,9} have primarily focused on the effects of severe thrombocytopenia and, with two exceptions,^{3,20} have not examined the effects of lesser degrees of thrombopoietic perturbation. Furthermore, relatively few studies have extensively characterized the temporal sequence and duration of the response of megakaryocytes or their precursors to acute or chronic perturbation of the circulating platelet mass.^{1,3,6,10,12,13} In addition, only one study has simultaneously examined the changes in megakaryocytes and circulating platelets in a serial fashion throughout the entire period of altered thrombopoiesis.¹

Previous efforts have been hampered by the lack of a suitable experimental model and techniques with which to measure the effects of perturbed thrombopoiesis on all three cellular compartments of thrombopoiesis: circulating platelets, recognizable megakaryocytes, and morphologically unrecognizable precursor cells. Advances in platelet isolation,²¹ analysis of megakaryocyte DNA content,⁹ and cultures that support the growth of megakaryocyte colonies from colony-forming cells²² now make more feasible a unified approach to the study of thrombopoiesis.

We have established a murine model and techniques to

extend the study of the regulation of thrombopoiesis. Previous reports by one of us have described the extended effects of acute and chronic, severe thrombocytopenia on Meg-CFC.^{10,11} The present report describes our use of a murine model to serially examine the effects of immune mediated thrombocytopenia of variable severity and duration on the ploidy distribution of bone marrow megakaryocytes and on the mean platelet volume (MPV) of circulating platelets.

MATERIALS AND METHODS

General techniques. C57/BL mice, with intact spleens, were used for all studies. Thrombocytopenia was induced by the intraperitoneal injection of guinea pig antimouse platelet serum (GP-PAS) that had been adsorbed against erythrocytes and leukocytes as previously described.¹⁰ Variable levels of thrombocytopenia were produced by adjusting the administered dose of GP-PAS; the level of thrombocytopenia was defined by the platelet count 12 or 24 hours

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after administration of GP-PAS. Due to interindividual variation in response to GP-PAS, cohorts of animals with a uniform degree of thrombocytopenia were produced by the following procedure. A large group of animals were injected with a dose of GP-PAS calculated to produce the desired level of thrombocytopenia based upon prior experience. At 12 or 24 hours after antiserum injection, platelet counts were performed on blood samples obtained from the retroorbital venous plexus, and collected into EDTA- and heparin-coated capillary tubes (Drummond Scientific Co, Broomall, PA). Platelet counts were performed by using a described technique²³ with an electronic particle counter (Coulter Electronics, Hialeah, FL). The animals were grouped for further studies according to their platelet levels.

Collection of bone marrow cells. At designated time points, mice were sacrificed by cervical dislocation, and the bone marrow was flushed from the femurs and humeri with a small volume of CATCH medium¹⁴ supplemented with sodium citrate (0.38%), prostaglandin E₁ (PGE₁) (1.0 µg/mL), horse serum (5%), and DNAase (30.0 Kunitz U/mL) into a larger volume of the same supplemented medium. Bone marrow was harvested and pooled from two animals for each flow cytometric analysis. A total nucleated cell count was determined by counting the diluted cell suspension with a Coulter S Plus IV particle counter (Coulter). After counting, the cells were divided into two aliquots containing 2 to 4 × 10⁷ cells each in 2 to 5 mL of supplemented CATCH medium. The cells were centrifuged at either 70 or 350 g for five minutes at 23°C, the supernatant fraction was aspirated, and the cells were resuspended in 100 µL of supplemented CATCH medium.

Identification of megakaryocytes and measurement of megakaryocyte DNA. Cells were defined as megakaryocytes and electronically selected from the total nucleated bone marrow cell population by a modification of the two-color fluorescence-activated flow cytometry technique originally described by Jackson and coworkers.⁹ Megakaryocytes were identified by labeling with a cell class-specific probe directed against multiple surface constituents. The probe used in these experiments consisted of a high-titer, polyclonal rabbit antimouse platelet serum (R-PAS) that had been adsorbed against mouse erythrocytes and leukocytes.

The potency and specificity of this reagent was evaluated by the following methods. First, the antiserum was assayed in a fluorescence-activated flow assay to determine the potency of binding to isolated mouse platelets and to establish the maximum dilution sufficient to produce adequate platelet labeling. This assay technique is based on our described method for the measurement of platelet-associated immunoglobulin.²⁴ Serial dilutions of the R-PAS were incubated with 5 × 10⁶ mouse platelets, and the binding of antibody was detected by using a second-step reagent consisting of biotin-conjugated goat antirabbit immunoglobulin Fab 2' fragment (GAR-Fab 2') (Dako Laboratories, Santa Barbara, CA) directed against all major heavy chain classes. The fluorescence detection system consisted of avidin-conjugated fluorescein. Labeled cells were analyzed in a Becton Dickinson Research Analyzer (Becton Dickinson, Mountain View, CA) as described.²⁴

The specificity of the R-PAS reagent was evaluated by an immunoblot assay with solubilized murine platelet proteins as the target antigens by utilizing our recently described method for human platelets.²⁵ The cell class specificity of the R-PAS reagent for murine megakaryocytes was also evaluated by examining the binding of R-PAS to murine bone marrow cells by light microscopy with immunocytochemical techniques.²⁶ Using these assay systems, we determined that a 1:4,000 dilution of the R-PAS in phosphate-buffered saline (pH 7.4) produced maximal platelet surface labeling. The immunoblot analyses demonstrated that the R-PAS reagent was active against multiple murine platelet proteins ranging from less than 45 kilodaltons (kd) to more than 300 kd, the resolving limits of

the gel system. The most intense labeling occurred against the region of the gel that contained the platelet glycoprotein IIb/IIIa complex. Microscopy with immunoperoxidase-labeled reagents demonstrated no reactivity of the R-PAS against nonmegakaryocytic bone marrow cells, whereas megakaryocytes of all three maturational stages³ were labeled by the polyclonal R-PAS.²⁶

Based upon the preceding observations, the following conditions were used for labeling of megakaryocytes in the two-color fluorescence DNA assay. An aliquot containing 2 to 4 × 10⁷ washed bone marrow cells was incubated in 900 µL of a 1:4,000 dilution of R-PAS for 60 minutes at 4°C. An identical aliquot of bone marrow cells was incubated with a 1:4,000 dilution of nonimmune, normal rabbit serum (NRS) to serve as a negatively stained control sample. From this point, the NRS control and the R-PAS-labeled aliquots were treated similarly. The cells, which had been incubated with either R-PAS or NRS, were washed three times with cold CATCH medium supplemented with 0.38% citrate and PGE₁ (1.0 µg/mL) (CATCH-PGE-citrate), suspended in 100 µL of CATCH-PGE-citrate medium, and then labeled with 200 µL of a 1:20 dilution of biotin-conjugated GAR-Fab 2' for 60 minutes at 4°C. Subsequently, the cell aliquots were washed three times with cold CATCH-PGE-citrate medium, resuspended in 100 µL of CATCH-PGE-citrate medium, and incubated for 30 minutes at 4°C with 400 µL of a 1:20 dilution of avidin-fluorescein isothiocyanate (FITC; 250 µg/mL, Becton Dickinson). After fluorescein labeling, the cells were washed three times with cold CATCH-PGE-citrate medium and resuspended in 0.67 mL of hypotonic citrate-saline buffer (100 mOsm/L) containing 1% bovine serum albumin (BSA) and propidium iodide (50 µg/mL). The 100-mOsm/L hypotonic solution permitted adequate penetration of the cells by propidium iodide for DNA staining with preservation of megakaryocyte cellular morphology. Cellular morphology was evaluated by microscopic examination of cytocentrifuge preparations of samples of the bone marrow cell suspensions stained with modified Romanowsky stain.²⁷ The cells were maintained in the hypotonic propidium iodide solution at 4°C for two hours to permit DNA staining by propidium iodide, treated with freshly prepared RNase (50 µg/mL) for 30 minutes at 23°C, and filtered through nylon mesh (75-µm pore diameter) immediately prior to fluorescence-activated cell analysis. Cell recovery after propidium iodide staining was determined for a number of bone marrow samples (N = 6) by performing a second total nucleated cell count. Total nucleated cell recovery averaged 56.7% ± 6.3% (SD).

The fluorescein and propidium iodide channels of the flow cytometer (Becton Dickinson FACS Research Analyzer) were aligned by using 5.76-µm beads. Fluorescence activation was produced with the 488-nm line of a high-pressure Hg light source. The analyzer was equipped with a 75-µm orifice, and the following photomultiplier (PMT) conditions were used: fluorescein channel PMT = 250 V, log scale; propidium iodide channel PMT = 250 V, log scale. The data were accumulated in a single-parameter mode and stored on a hard disk for subsequent analysis. Bone marrow cells were analyzed at a flow rate of 200 to 400 cells per second without sample agitation.

Electronic selection of megakaryocytes from the unfractionated bone marrow cell population requires the positioning of an electronic fluorescein gate, or "green gate," to discriminate the megakaryocyte subpopulation from the total bone marrow cell population. This selected subpopulation was then analyzed for DNA content by propidium iodide staining in the second analyzer channel. Instead of setting the fluorescein gate at the point where 99% of the signals from cells labeled with NRS were excluded as in the method of Jackson et al.,⁹ we analyzed the total cell population after labeling with both the NRS and R-PAS reagents and then selected a point that was beyond the 99% exclusion level and at the nadir of the fluorescence distribution between the unlabeled and labeled cell populations (Fig 1A). This alternative green gate, subsequently

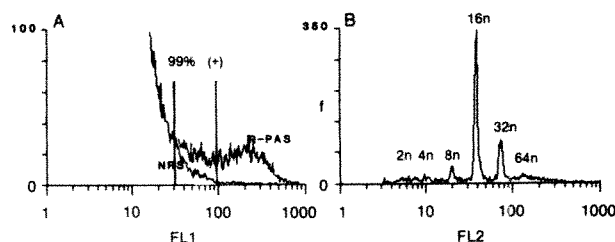


Fig 1. (A) The fluorescence distribution of unfractionated bone marrow cells is shown after labeling with the megakaryocyte-specific antisera (R-PAS) or NRS. Note that the initial portions of these two distributions overlap. The abscissa indicates fluorescence intensity (FL 1-logarithmic scale), and the ordinate indicates the cell number (f) at full scale. The leftmost vertical marker (99%) indicates the 99% electronic exclusion gate that selects the 1% brightest cells, whereas the marker to the right (+) indicates the nadir between the unlabeled population (to the left) and the labeled population (to the right) of bone marrow cells. The latter marker is referred to as the positive green gate marker. The small fraction of cells to the right of the positive green gate marker was designated as the megakaryocyte population and was subjected to DNA analysis as shown in B. (B) The distribution of the DNA content is shown for the selected cell population, which has been electronically isolated in A on the basis of surface fluorescence. The abscissa indicates fluorescence intensity (FL 2-logarithmic scale) and the ordinate is cell number (f) at full scale. The peaks are as follows from left to right: 2N, 4N, 8N, 16N, 32N, and 64N. One thousand selected cells were analyzed to obtain the megakaryocyte ploidy distribution.

termed the "positive green gate," is a more conservative gating point than the 99% exclusion point and produced DNA distributions with discrete separations of the different megakaryocyte ploidy classes (Fig 1B).

The analyzer threshold was adjusted by using the propidium iodide channel to exclude subcellular debris and to include all cells with a DNA content of 2N or greater. The megakaryocyte DNA content was measured in all cells with fluorescence greater than the positive green gate marker and with a DNA content of 2N or greater. For determination of the megakaryocyte DNA distribution, each sample was analyzed for a time period sufficient to accumulate between 3,000 and 4,000 megakaryocytes, as defined by cell surface labeling. The major fluorescent peak for DNA of the nonmegakaryocyte cells was determined in each analysis and was used as the 2N standard to set windows of equal width for the 2N to 128N ploidy classes.

To evaluate the possibility that intraperitoneal administration of GP-PAS could result in direct *in vivo* binding of antibody to megakaryocytes as well as to platelets, we determined whether our assay system for the detection of megakaryocytes using biotin-conjugated GAR-Fab 2' could also detect GP-PAS bound to mouse platelet or megakaryocyte surface proteins. When mouse platelets were incubated with a 1:4,000 dilution of GP-PAS instead of R-PAS, we observed labeling with the biotin-conjugated GAR-Fab 2' fragments that was equal to that observed with the R-PAS first-step reagent. This indicates that if significant direct *in vivo* labeling of mouse megakaryocytes occurred it could potentially be detected by the second-step reagent in the two-color assay system.

Measurement of MPV. Blood for the measurement of the MPV was obtained by puncture of the retroorbital venous plexus with EDTA- and heparin-coated capillary tubes. Seventy microliters of blood was drawn into each of two glass capillary tubes, and the blood was immediately added to 330 μ L of buffered anticoagulant solution containing the following final concentrations: sodium citrate, 13 mmol/L; EDTA, 1 mmol/L; PGE₁, 1 μ g/mL; and theophylline, 0.18

mg/mL. Platelet volume studies from severely thrombocytopenic animals required the collection of up to seven capillary tubes per animal. Under these conditions, three capillary tubes were added to each 330- μ L volume of buffered anticoagulant solution. Samples from two to five mice, either controls or treated animals, were pooled and layered over a discontinuous arabinogalactan gradient composed of 10% (wt/wt) and 20.5% (wt/wt) layers as previously described for rabbit platelets.²¹ The gradients were centrifuged at 20,000 g for ten minutes at 23°C to separate the platelets from the plasma proteins, erythrocytes, and granulocytes. Lymphocytes and monocytes exhibit a density distribution that overlaps that of platelets; since these leukocytes cosediment with platelets, they were electronically excluded from the platelet size distribution by setting an upper volume threshold.²¹ The MPV was measured with an electrical impedance cell analyzer (Particle Data, Inc, Chicago) equipped with a 48- μ m orifice tube, a logarithmic analyzer, and a PDP 11A computer. This instrument was calibrated with 2.02- μ m-diameter latex beads as previously described.²¹ The MPV was measured as the geometric mean of the platelet volume distribution curve and expressed in femtoliters. Contaminating erythrocytes were excluded by use of an electronic upper threshold, and a Gaussian curve-fitting program was utilized to complete the upper end of the platelet volume distribution curve.

Statistical analysis. The means and SD of the bone marrow megakaryocyte ploidy class distributions, platelet counts, and MPVs were computed by standard methods. Statistical comparisons of the MPV between the normal murine population and the experimental groups were performed with a standard *t* test.²⁸ Statistical comparisons of the megakaryocyte ploidy distributions between the normal population and the experimental groups were performed with the Mann-Whitney rank test.²⁹

RESULTS

Megakaryocyte DNA distribution in mice with normal platelet levels. The distribution of DNA content in murine bone marrow megakaryocytes was measured by using bone marrow harvested from normal animals. The positive green gate marker technique produced megakaryocyte DNA distributions with clear separations between the ploidy classes (Fig 1B). Under our operating conditions, the observed

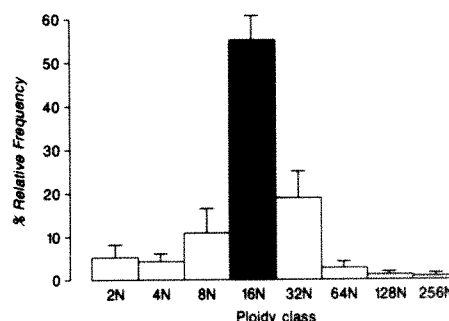


Fig 2. The frequency distribution of megakaryocyte DNA content in the bone marrow of normal mice is shown. Ploidy classes from 2N to 256N are indicated on the abscissa. The relative mean frequency of each ploidy class ± 1 SD is indicated as a percentage of the total megakaryocyte population on the ordinate. The 16N cell class, the normal modal class, is identified by the solid black bar for reference. Twenty-five analyses were performed with the positive green gate technique; each consisted of the pooled bone marrow from two animals. The average number of megakaryocytes analyzed in each experiment to determine the ploidy distribution was $3,413 \pm 1,273$ (SD).

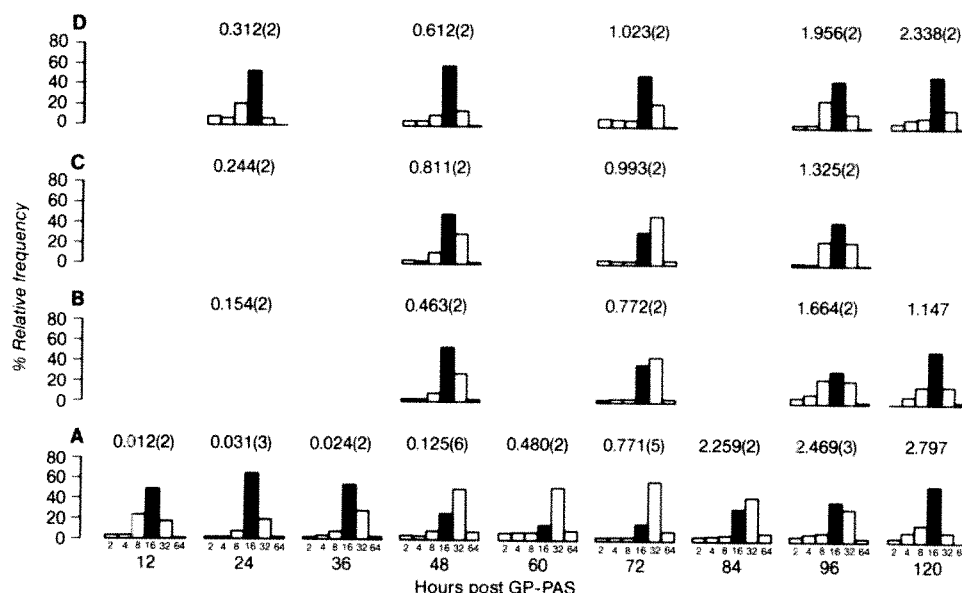


Fig 3. The frequency distribution of megakaryocyte DNA content after the induction of various degrees of acute thrombocytopenia is shown. Each sample consisted of the pooled bone marrow from two animals. The abscissa indicates the ploidy class from 2N to 64N, and the ordinate indicates the relative frequency (in percentage) of the various ploidy classes. The time after GP-PAS administration at which bone marrow samples were obtained is indicated in hours along the lower abscissa and is the same for each experimental series. The mean platelet count ($\times 10^6/\mu\text{L}$) of the animals studied at each time point is indicated above the individual histograms. The number of experiments performed at each time point is indicated in parentheses, except where only a single experiment was performed. Absence of a ploidy histogram indicates that a DNA study was not performed at that time point, but the mean platelet count is indicated. The mean platelet counts ($\times 10^6/\mu\text{L}$) 24 hours after GP-PAS administration were as follows: A, 0.031; B, 0.154; C, 0.244; D, 0.312. The normal modal ploidy class was 16N. In each histogram, the relative frequency of the 16N cell class is identified by the solid black bar.

modal megakaryocyte ploidy class was 16N (Fig 2). We calculated the fraction of cells in the bone marrow cell suspension that were immunologically identified as megakaryocytes. Under these conditions, this measurement provides an approximate estimate of megakaryocyte frequency in unfractionated bone marrow. The mean value for 25 experiments, each consisting of two animals, was $0.496\% \pm 0.35\%$ SD.

Effects of acute, severe thrombocytopenia on the distribution of megakaryocyte DNA and MPV. Acute, severe thrombocytopenia (platelet count reduction to $<0.05 \times 10^6/\mu\text{L}$, 5% of normal) 12 hours after PAS administration resulted in no detectable change in the distribution of megakaryocyte ploidy at 12, 24, or 36 hours after GP-PAS administration (Fig 3A). Although the frequency of the 32N class increased at 36 hours after GP-PAS administration, this difference was not statistically significant ($P = .07$). Forty-eight hours after administration of GP-PAS, a shift in the modal ploidy class from 16N to 32N was consistently observed (Fig 3A). The frequency of 64N cells also was significantly increased ($P < .001$) at 48 hours after induction of thrombocytopenia and remained increased until 96 hours. At 48 hours after GP-PAS administration, the platelet level ($0.125 \times 10^6/\mu\text{L}$) had begun to return toward normal (Fig 4). However, the shift in the modal ploidy class from 16N to 32N persisted until 84 hours after administration of GP-PAS. At 96 hours after administration of GP-PAS, the frequency of the 32N class was still increased ($P < .02$), but the 32N class was no longer the modal class. The megakaryocyte DNA distribution returned to normal 120 hours after PAS administration. Animals treated with

nonimmune guinea pig serum (NGPS) did not demonstrate any significant changes in megakaryocyte DNA distribution compared with untreated controls.

Bone marrow cells obtained from animals treated with GP-PAS and then exposed only to NRS *in vitro* did not show any evidence of *in vivo* megakaryocyte labeling, and labeling could only be demonstrated after *in vitro* exposure of the cells to R-PAS. This suggests that binding of intraperitoneally administered GP-PAS to native megakaryocytes was not present 24 hours after PAS administration, although it is possible that a small amount of binding could have occurred earlier or at a concentration that was below the detection threshold of the assay. Platelets isolated from animals 24 hours after GP-PAS administration also did not demonstrate guinea pig immunoglobulin on the surface by examination with immunoelectron microscopy.³⁰

MPV was measured from 8 to 96 hours after the induction of acute, severe thrombocytopenia (reduction of platelet count to less than $0.05 \times 10^6/\mu\text{L}$) (Table 1). MPV was significantly increased ($P < .001$) at eight hours after the administration of GP-PAS. The peak increment in MPV occurred at 24 hours, and MPV then slowly declined to the normal range at 96 hours. Importantly, MPV decreased continuously from the peak level, although it remained significantly elevated ($P < .001$) at each time point, as the platelet level increased dramatically during the 36- to 84-hour period after induction of acute thrombocytopenia (Table 1).

Effects of variable degrees of acute thrombocytopenia on megakaryocyte DNA distribution. The effect of the severity of thrombocytopenia on megakaryocyte DNA distri-

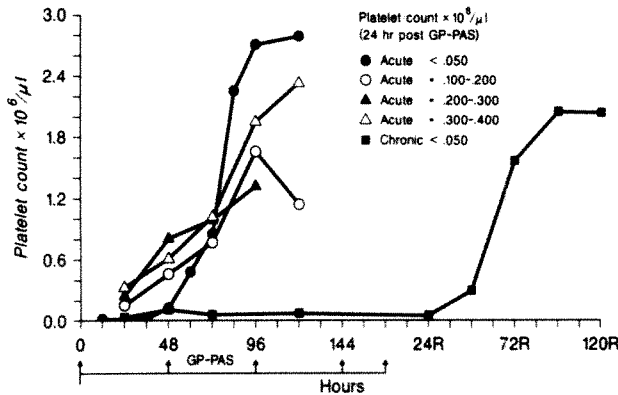


Fig 4. The time course for recovery of platelet levels after the induction of experimental thrombocytopenia is shown. The platelet count ($\times 10^6/\mu\text{L}$) is indicated on the ordinate and time (h) is indicated on the abscissa. Platelet counts during recovery from various degrees of thrombocytopenia were obtained from variable numbers of mice in each experimental group: 12 hours, 12; 24 hours, 6 to 24; 36 hours, 2; 48 hours, 2 to 8; 60 hours, 2; 72 hours, 2 to 11; 84 hours, 2; 96 hours, 2 to 8; 120 hours, 2 to 3. For the group with chronic thrombocytopenia the following numbers of animals were used at the respective time points during the initial treatment period: 24 hours, 30; 48 hours, 2; 72 hours, 8; 120 hours, 5. During the recovery phase from chronic thrombocytopenia the following numbers of animals were used to determine the platelet counts at the respective time points: 24 hours, 20; 48 hours, 6; 72 hours, 5; 96 hours, 5; 120 hours, 4. The following symbols denote the level of thrombocytopenia 24 hours after administration of GP-PAS: solid circle, $<0.05 \times 10^6/\mu\text{L}$; open circle, 0.100 to $0.200 \times 10^6/\mu\text{L}$; solid triangle, 0.200 to $0.300 \times 10^6/\mu\text{L}$; open triangle, 0.300 to $0.400 \times 10^6/\mu\text{L}$, and solid square, chronic thrombocytopenia of $<0.05 \times 10^6/\mu\text{L}$. The arrows below the abscissa indicate the times of GP-PAS administration during the chronic thrombocytopenia experiment. The large arrow indicates the initial GP-PAS injection in all experiments. The time points with the suffix R indicate the recovery phase of the chronic thrombocytopenia experiment.

bution was evaluated by adjusting the dose of GP-PAS to produce lesser degrees of thrombocytopenia. Animals were treated with various single doses of GP-PAS and placed according to the platelet count 24 hours after GP-PAS administration into one of three groups: 0.100 to 0.200×10^6 platelets/ μL , 0.200 to 0.300×10^6 platelets/ μL , and 0.300 to

Table 1. The Effect of Acute Severe Thrombocytopenia on MPV

Time	C	8	12	24	36	48	60	84	96
MPV	4.6	6.8*	5.9*	8.2*	7.3*	6.7*	5.8*	5.2†	5.1
N	25	4	11	8	8	4	10	10	10
PC	1.62	.02	.03	.02	.10	.16	.58	1.42	2.00
SD	0.40	0.01	0.02	0.03	0.03	0.06	0.19	0.70	1.26

NOTE. C indicates a normal control population. The MPV of the normal population, $4.6 \text{ fL} \pm 0.5 \text{ SD}$, was used for statistical comparisons with the treated population. The time after GP-PAS administration is indicated in hours, MPV is expressed in femtoliters, and the platelet count (PC) $\times 10^6/\mu\text{L}$ with the SD of the mean is shown. N represents the number of individual animals utilized at each time point to determine the platelet count. Blood from these animals was pooled to measure MPV at each time point in two separate experiments. MPV of control animals was obtained from individual animals. A separate group of animals was used for the ploidy analyses.

* $P < .001$.

† $P < .01$.

0.400×10^6 platelets/ μL . The megakaryocyte DNA distribution (Figure 3B to D) and the time course of recovery of the platelet level (Fig 4) were serially measured. Reduction of the platelet level to 0.100 to 0.200×10^6 platelets/ μL (mean, $0.154 \pm 0.037 \text{ [SD]} \times 10^6$ platelets/ μL ; range, 0.098 to 0.232×10^6 platelets/ μL) did not produce a shift in the modal ploidy class at 48 hours after GP-PAS administration (Fig 3B), the time point at which a ploidy shift was consistently observed after severe, acute thrombocytopenia (Fig 3A). However, at 48 hours after administration of GP-PAS, the relative frequency of 32N megakaryocytes increased to 28%, the upper limit of the normal range for the 32N class ($P < .05$). More importantly, at 72 hours after GP-PAS administration, there was a shift in the modal megakaryocyte ploidy class from 16N to 32N (Fig 3B). Therefore, the shift in ploidy class in response to acute thrombocytopenia, which was always observed at 48 hours after severe thrombocytopenia (platelet level, $<0.05 \times 10^6$ platelets/ μL), was delayed for an additional 24 hours when the stimulus of thrombocytopenia was less severe. At 96 hours after the induction of moderate thrombocytopenia (0.100 to 0.200×10^6 platelets/ μL), the modal ploidy class reverted to 16N, and a complete return to the baseline DNA distribution was observed 120 hours after GP-PAS injection. No significant increment in the 64N megakaryocyte class was observed at any time.

Reduction of the platelet level to 0.200 to 0.300×10^6 platelets/ μL (mean, $0.244 \pm 0.026 \text{ [SD]} \times 10^6$ platelets/ μL ; range, 0.227 to 0.296×10^6 platelets/ μL) also resulted in a delayed shift of the megakaryocyte DNA distribution similar to that observed with the preceding experimental group (Fig 3C). The 32N megakaryocyte class was not significantly increased at 48 hours after administration of GP-PAS ($P > .05$), and a shift in the modal ploidy class from 16N to 32N occurred at 72 hours. Additional experiments (Fig 3D) in which the platelet level was reduced to 0.300 to 0.400×10^6 platelets/ μL (mean, $0.354 \pm 0.059 \text{ [SD]} \times 10^6$ platelets/ μL ; range, 0.227 to 0.441×10^6 platelets/ μL) demonstrated no significant change in frequency of the 32N class at any time.

When very moderate thrombocytopenia (platelet count nadir of 0.400×10^6 platelets/ μL at 16 hours after GP-PAS injection) was produced, a highly significant change in MPV occurred as early as 12 hours after administration of GP-PAS (Table 2, group B) and persisted until 144 hours after GP-PAS administration. It should be noted that the platelet count nadir was probably missed for animal group A (Table 2) due to the long sampling interval from four to 24 hours after GP-PAS injection. The increase in platelet volume during this experiment reached a peak 24 hours after administration of GP-PAS, at which time the megakaryocyte ploidy distribution was entirely within the normal range in response to slightly greater thrombocytopenia (Fig 3D). Thus, an effect on platelet volume was produced by a level of thrombocytopenia that did not alter megakaryocyte DNA distribution during the 96-hour period after moderate thrombocytopenia (Fig 3D).

Effects of chronic, severe thrombocytopenia on megakaryocyte DNA distribution. Chronic, severe thrombocytopenia was induced by a reduction of the platelet level to less

Table 2. The Effect of Acute Moderate Thrombocytopenia on MPV

Group A						
Time	C	4	24	48	72	144
MPV	4.1	5.3*	6.3†	6.3†	5.7†	5.6†
PC	1.24	1.1	0.95	0.85	1.65	2.50
SD	0.11	0.56	0.28	0.17	0.68	1.13
Group B						
Time	C	12	16	36	64	108
MPV	4.1	5.7†	5.6†	5.6†	6.3†	5.6†
PC	1.24	0.65	0.40	0.75	1.2	2.2
SD	0.11	0.17	0.14	0.19	0.59	1.16

NOTE. Two animal groups (A and B) were used to obtain serial measurements of MPV after the induction of thrombocytopenia. A separate group of animals was used for the ploidy analyses. Group A was studied at 4, 24, 48, 72, and 144 hours, and group B was studied at 12, 16, 36, 64, and 108 hours after GP-PAS administration. MPV is expressed in femtoliters, and the platelet count $\times 10^6/\mu\text{L}$ (PC) is indicated with the SD of the mean. MPV and platelet count data at each time point represent mean values from eight individual animals. The MPV of 22 normal control animals (C) was $4.1 \text{ fL} \pm 0.5 \text{ SD}$. Statistical comparisons were made with this control population.

* $P < .005$.

† $P < .001$.

than 0.05×10^6 platelets/ μL and was then maintained at approximately that level by repeated administration of GP-PAS every other day for a seven-day period (Fig 4). After 120 hours of sustained, marked reduction in platelet levels (Fig 4), the bone marrow megakaryocyte DNA distribution was determined (Fig 5). The modal ploidy class was 32N, and the frequency of the 64N class was significantly increased ($P < .05$). Twenty-four hours after discontinuation of chronic GP-PAS administration, the mean platelet level ($\times 10^6/\mu\text{L}$) of the entire group of mice ($n = 21$) was 0.033 ± 0.016 (SD) with a range from 0.019 to 0.064. At this time, eight days after initiation of thrombocytopenia, the frequency of 16N and 32N cells was approximately equal, and the frequency of the 32N cells remained significantly increased compared with normal ($P < .05$) (Fig 5). Forty-eight hours after the last GP-PAS dose, 32N cells again constituted the modal class, and the 64N cells constituted 24.9% of the megakaryocyte population. This latter change was the largest increment in 64N cells observed in response to thrombocytopenia and was significant compared with the normal DNA distribution ($P < .05$). As platelet recovery occurred (Fig 4), the frequency of both 32N and 64N cell classes declined (Fig 5), although both remained significantly elevated until 120 hours after the last GP-PAS dose, at which time the distribution returned to normal.

DISCUSSION

Previous studies have provided limited and conflicting information concerning the regulation of thrombopoiesis after experimental thrombocytopenia. Ebbe et al³ demonstrated an increase in rat megakaryocyte volume that reached a maximum 48 hours after a 92% reduction of the platelet level by exchange transfusion. Extreme thrombocytopenia (99.9% reduction of the platelet level induced by platelet antiserum) produced a larger increase in mega-

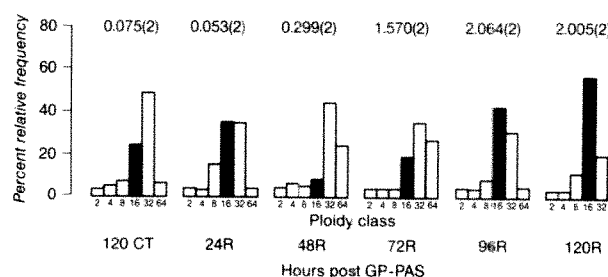


Fig 5. The frequency distribution of megakaryocyte DNA content after the induction of chronic, severe thrombocytopenia (platelet count $< 0.05 \times 10^6/\mu\text{L}$) is shown. Studies were performed after 120 hours of chronic thrombocytopenia (120 CT time point) and at 24, 48, 72, 96, and 120 hours of the recovery phase after cessation of administration of GP-PAS. The recovery time points are denoted by the suffix R. The ploidy classes from 2N to 64N are indicated along the abscissa, and the relative frequency of each class is indicated as a percentage of the total megakaryocyte population on the ordinate. The 16N cell class is identified by the solid black bar for reference. The mean platelet count ($\times 10^6/\mu\text{L}$) for the animals used for the ploidy distribution analyses at each time point is indicated above the individual histograms, and the number of experiments performed at each time point is indicated in the parentheses.

karyocyte cell volume than observed after a 92% reduction in the platelet level. Other studies by Harker,⁴ also using rats, extended these observations to demonstrate a parallel shift in megakaryocyte "nuclear number" as well as volume after four days and ten days of an 80% reduction in the platelet level. However, Harker did not directly determine the megakaryocyte DNA content. Studies by Penington and Olsen⁶ and Odell and coworkers¹ using direct measurement of megakaryocyte DNA found the normal rat megakaryocyte modal ploidy class to be 16N and observed that induction of acute, severe thrombocytopenia resulted in a shift of the modal class from 16N to 32N 48 hours after reduction in the platelet level. Odell et al¹ described an early increase in platelet size 18 hours after induction of thrombocytopenia and noted that the maximum increase in platelet size preceded the peak increase in both megakaryocyte volume and ploidy distribution by 30 hours.

After six days of variable, chronic thrombocytopenia, Martin et al⁷ observed a marked increase in total megakaryocyte cell volume, cytoplasmic volume, and nuclear volume. MPV increased by approximately 10% compared with the control during the first four days and then rapidly increased by an additional 30% at the sixth day. In a second paper,⁸ these workers reported a shift from the normal modal megakaryocyte ploidy class of 16N to 32N after 24 hours of thrombocytopenia (90% reduction in platelet level); a similar shift also was observed after six days of chronic thrombocytopenia. In a third study, Trowbridge and Martin² produced an 86% reduction in the platelet level of rabbits and observed a 27% increase in MPV six hours after the induction of thrombocytopenia. However, they observed no change in the modal megakaryocyte ploidy class from their normal control value of 32N either two hours or 24 hours after the onset of thrombocytopenia, although there was an increase in the relative frequency of the 32N class. Martin et al concluded that thrombocytopenia results in an increase in megakaryo-

cyte ploidy and cytoplasmic volume with subsequent production of larger platelets.⁸

Using a modified two-color technique based on the method of Jackson et al,⁹ we have observed that the modal megakaryocyte ploidy class in mice with normal platelet levels is 16N. Our megakaryocyte ploidy distributions are similar to those of Jackson et al although the use of the more conservative megakaryocyte selection gate reduced the observed frequency of 2N and 4N megakaryocytes. These differences may be partially due to species variation as well. Our determinations of the normal murine megakaryocyte ploidy distribution are in general agreement with the earlier observations of Odell et al and Penington and Olsen using the Feulgen microdensitometric technique with rat bone marrow smears,^{6,31} those of Worthington et al³² with a single-color flow cytometric technique, and those of Mayer et al³³ using immunofluorescent identification of megakaryocytes followed by Feulgen staining to measure the DNA content. In each of these studies^{6,9,31-33} the 2N and 4N classes were infrequent, 16N was the modal class, and 8N cells ranged in frequency from 8.1% to 23.0%. The results from these investigations and those of the present study do not agree with the observations of other workers who have used single-color fluorescence-activated flow cytometry and found that 8N is the most common ploidy class.^{14,15} Jackson et al suggest that this discrepancy may be due in part to failure of the single-color flow technique to exclude cell aggregates,⁹ a problem that is minimized by the two-color technique.

The two-color technique not only clearly resolves the individual ploidy classes but also is advantageous because of the large numbers of cells analyzed and the use of a megakaryocyte selection technique that is independent of morphology. Immunologic identification of megakaryocytes could potentially underestimate the number of megakaryocytes if the megakaryocyte-specific probe failed to detect a portion of the megakaryocyte population. However, the probe used in the present studies was found to be sensitive to a broad spectrum of murine platelet proteins, which indicates that there should be broad sensitivity for megakaryocyte proteins as well. The studies of Rabellino et al³⁴ demonstrate that the small, immature human megakaryocytes express the major platelet glycoproteins Ib, IIb, and IIIa. Jackson has demonstrated good correlation between the identification of rat megakaryocytes with PAS and detection of acetylcholinesterase.³⁵ The recent report by Ishibashi et al³⁶ also provides evidence that immature, morphologically unrecognizable megakaryocytes are identified by a monoclonal antibody directed against the glycoprotein IIb/IIIa complex. Since it is possible that the electronic gating technique could exclude low-ploidy megakaryocytes that contain low levels of surface fluorescence, we performed additional experiments using dual-parameter analysis in which the gate was further reduced below the standard positive green gate. This less-restrictive gating technique did not significantly increase the frequency of 2N, 4N, or 8N class megakaryocytes (data not shown). Our estimate of the frequency of megakaryocytes in normal murine bone marrow (0.496%) is in approximate agreement with earlier immunofluorescent microscopic determinations using murine marrow³³ and is in the range of

estimates obtained by a number of workers using rodents and a variety of techniques.³⁷⁻³⁹

We have demonstrated that the modal ploidy class shifts from 16N to 32N 48 hours after the induction of acute, severe thrombocytopenia. The shift is consistently initially observed 48 hours after administration of antiserum, persists until 84 hours after GP-PAS administration, and then reverts to normal 120 hours after the onset of thrombocytopenia. Jackson et al⁹ also observed a shift in the megakaryocyte ploidy distribution of rats 48 hours after the onset of thrombocytopenia, but the more frequent sampling in the present study clearly documented that no shift in the modal ploidy class was observed until the 48-hour time point.

The degree of thrombocytopenia in the study of Ebbe et al³ at which a significant increase in megakaryocyte cytoplasmic volume was observed is similar to that in our study of moderate thrombocytopenia (0.300 to 0.400×10^6 platelets/ μL 24 hours after GP-PAS administration). Under these conditions, we observed no significant change in the megakaryocyte ploidy distribution during the entire five-day period of observation (Fig 3D). Furthermore, MPV increased significantly 12 hours after GP-PAS administration in mice, with even a lesser reduction of platelet levels (Table 2). After acute severe thrombocytopenia (platelet count reduction to less than 0.05×10^6 platelets/ μL after administration of GP-PAS), we observed an increase in MPV 40 hours prior to a significant effect on the megakaryocyte ploidy distribution and 28 hours before the initial increase in the proportion of 32N megakaryocytes. Our observations indicate that the increases in platelet volume after acute thrombocytopenia clearly precede and therefore are dissociated from major changes in the megakaryocyte ploidy distribution; these increases constitute the initial response to reduced levels of circulating platelets, even when the degree of diminution of the circulating platelet mass is insufficient to produce a detectable alteration in megakaryocyte DNA content.

Moderate degrees of thrombocytopenia (reduction in platelet levels to approximately 10% to 20% of the normal level) resulted in a more delayed shift of the ploidy distribution. Very moderate thrombocytopenia (reduction in platelet level to 40% of normal) did not produce a shift in the modal ploidy class even as late as 96 hours after GP-PAS administration, although platelet volume was increased. These results suggest that the effect of thrombocytopenia on nuclear endoreduplication is modulated by the magnitude of the stimulus. Pertinently, Odell and Shelton⁴⁰ reported that endomitosis was not significantly increased in megakaryocytes unless platelet levels were reduced to less than 50% of normal.

The present studies of chronic, severe thrombocytopenia indicated that the megakaryocyte ploidy distribution remained significantly shifted during persistent thrombocytopenia and that the recovery phase was associated with a more marked increase in the 64N cell class than was observed during recovery from acute thrombocytopenia (Fig 5). This increased proportion of 64N class megakaryocytes may also explain the more rapid rate of platelet recovery observed in response to chronic thrombocytopenia as com-

pared with that after acute, severe thrombocytopenia (Fig 4). It is of interest that the megakaryocyte DNA distribution returned to normal 120 hours after discontinuation of platelet antiserum regardless of the magnitude or duration of the shift in the megakaryocyte DNA distribution. The present studies are consistent with the earlier results of Penington and Olsen.⁶ The lesser increase in the proportion of 32N megakaryocytes observed by Penington and Olsen⁶ is likely

due to their less frequent schedule of platelet antiserum administration, which probably did not produce as severe and consistent a period of prolonged thrombocytopenia as did our administration of antiserum at 48-hour intervals (Fig 4). Overall, our data demonstrate that the magnitude and temporal sequence of alterations that follow the induction of thrombocytopenia depend upon the magnitude and duration of the thrombocytopenic stimulus.

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Clonal Immunoglobulin Gene Rearrangements in Tissues Involved by Hodgkin's Disease

By Marja G.L. Brinker, Sibrand Poppema, Charles H.C.M. Buys, Wim Timens, Jan Osinga, and Lydia Visser

The nature of Reed-Sternberg cells, the abnormal cells of Hodgkin's disease, is controversial. Morphological and immunologic marker studies suggested different cells of origin. To investigate a possible B or T cell origin, immunoglobulin and T cell receptor gene analyses were performed on tissues from 11 patients in early and late stages of Hodgkin's disease. In addition, the immunologic marker patterns of the Reed-Sternberg cells were determined.

Rearrangements of immunoglobulin heavy- and light-chain genes were detected in tissues from five patients, particularly in late stages of the disease when lymphocyte depletion had occurred. No rearrangements of T cell receptor genes were found. The results indicate that clonal immunoglobulin gene rearrangements can be detected in tissues involved by Hodgkin's disease.

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THE NATURE OF REED-STERNBERG cells, the abnormal cells of Hodgkin's disease, is controversial. There is no formal proof that Reed-Sternberg cells in Hodgkin's disease constitute a monoclonal population. T cells, B cells, and macrophages have been implicated as the cells of origin of Reed-Sternberg cells.¹⁻³ Cell lines derived from tissue or pleural fluid involved by Hodgkin's disease may contribute to the solution of this problem; however, it is usually not possible to obtain absolute proof for the Reed-Sternberg cell origin of such cell lines.⁴⁻⁶ Because Reed-Sternberg cells do not produce detectable amounts of immunoglobulins, no firm evidence for a B cell origin is available. On the other hand, no reactivity with specific anti-T cell or antimacrophage antibodies could be demonstrated.⁷ Recently, some monoclonal antibodies prepared against a true histiocytic cell line were reported to be reactive with Reed-Sternberg cells.⁸ Reactivity with antibodies like Ki-1⁹ and Leu M1¹⁰ does not indicate a particular cell of origin of Reed-Sternberg cells. In some cases Reed-Sternberg cells are reactive with monoclonal anti-B cell reagents,¹¹⁻¹³ and in lymphocyte-predominant Hodgkin's disease the presence of J chains has been demonstrated in lymphocytic and histiocytic (L&H)-type Reed-Sternberg cells, thereby indicating the B cell origin of this type of Reed-Sternberg cell.^{14,15}

During the past few years, it has been shown that rearrangements of immunoglobulin genes can be demonstrated in clonal populations of B cells not or not yet producing immunoglobulin¹⁶ and, similarly, that rearrangements of T cell receptor genes can be demonstrated in clonal T cell populations.¹⁷ Here we describe the results of immunoglobulin and T cell receptor gene analysis in tissue samples of 11 patients with Hodgkin's disease.

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MATERIALS AND METHODS

Tissue selection. Tissue specimens of 11 patients with Hodgkin's disease were studied. In all cases the original diagnosis was made on hematoxylin and eosin (H&E)-stained paraffin or glycol-methacrylate-embedded lymph node tissue sections. The cases were selected for the presence of relatively high numbers of Reed-Sternberg cells. In all cases the slides were reviewed by three expert lymphoma pathologists. Histological subtyping was performed according to the Rye classification.¹⁸ Autopsy tissue was available in patients 3, 4, and 5, who had clinical stage IV nodular sclerosis or the mixed-cellularity type of Hodgkin's disease with poor responses to chemotherapy. At autopsy in 1977, 1978, and 1984, respectively, we obtained at least 20 frozen tissue blocks of macroscopically vital tumor tissue in lung and liver, and these blocks were kept at -80°C. Frozen tissue blocks for the present study were selected on the basis of H&E-stained frozen tissue sections to ensure a high concentration of Reed-Sternberg cells and mononuclear variants and minimal necrosis or fibrosis. In patient 2 involved lymph node as well as spleen was available, and in patient 3 involved spleen before treatment and heavily involved lung tissue at autopsy could be studied. Patient data are summarized in Table 1.

DNA isolation and gene analyses. DNA was isolated from the selected tissue blocks. As controls for germ line patterns, human placentas were used that were representative for the reported restriction fragment polymorphism in the λ gene locus.¹⁹ Minced

Table 1. Patient Data

No.	Sex	Age (yr)	Tissue	Type	Source
1	M	18	Lymph node	NS	Pretreatment
2	M	36	Lymph node	NS	Relapse 11 mo after diagnosis
			Spleen	NS	Relapse 11 mo after diagnosis
3	M	20	Spleen	NS	Pretreatment
			Lung	NS/LD	Autopsy 14 mo after diagnosis
4	M	22	Liver	NS/LD	Autopsy 18 mo after diagnosis
5	M	20	Lung	MC/LD	Autopsy 9 mo after diagnosis
6	F	41	Lymph node	NS	Pretreatment
7	F	30	Lymph node	NS	Pretreatment
8	M	32	Lymph node	NS	Relapse 12 mo after diagnosis
9	F	63	Lymph node	NS	Pretreatment
10	M	41	Lymph node	NS	Pretreatment
11	M	13	Lymph node	NS	Pretreatment

Abbreviations: NS, nodular sclerosis subtype; MC, mixed cellularity subtype; LD, lymphocyte depletion.

Table 2. Source of Antibodies and Reactivity With Reed-Sternberg Cells in Tissues With Clonal Rearrangements

Antibody	MW	Source	Reactivity						
			LN1	LN2	Spleen 2	Spleen 3	Lung 3	Liver 4	Lung 6
B1 (pan-B cell) ²⁸	p 35	Nadler	+	±	±	—	—	+	—
B2 (C3d receptor) ²⁹	p 140	Nadler	—	—	—	±	±	—	—
B4 (pan-B cell) ³⁰	p 45	Coulter	+	±	±	—	—	+	—
Ki-1 (R-S cell) ⁹	p 116, 126	Stein	±	+	+	+	+	±	+
Leu M1 (myeloid + R-S cell) ¹⁰	p 180, 110	B&D	+	+	+	+	+	—	+
Tac (interleukin 2 receptor) ³¹	p 60	Waldman	+	+	+	+	+	+	+
OKT9 (transferrin receptor) ³²	p 94	Ortho	+	+	+	+	+	+	+
OKI1 (HLA-DR antigen) ³³	p 32	Ortho	+	+	+	+	+	+	+
OKT11 (E rosette receptor) ³⁴	p 55	Ortho	±	—	—	—	—	—	—
OKT3 (T cell receptor assoc) ³⁵	p 22, 28	Ortho	±	—	—	—	—	—	—

Abbreviation: R-S, Reed-Sternberg; assoc, associated; p, protein.

Reactivity key: +, majority of Reed-Sternberg cells are positive; ±, minority of Reed-Sternberg cells are positive; —, Reed-Sternberg cells—negative.

tissues were incubated at 37°C overnight in 10 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, and 1 mmol/L EDTA containing 1% (wt/vol) sodium dodecyl sulfate (SDS) and 100 µg proteinase K/mL buffer. High-molecular weight DNA was obtained after extractions with phenol, incubation with DNase-free RNase A, further extraction with phenol/chloroform (1:1, vol/vol), and precipitation at –20°C with 2 vol of ethanol and 1/10 vol of a 3-mol/L sodium acetate solution. Digestions were carried out with *Eco*RI or *Bam*HI in the buffers recommended by the supplier (Boehringer, Mannheim, Göttingen) at 37°C for three to four hours by using 2.5

U of enzyme/µg of DNA. Digestion products were separated by size on 0.6% agarose gels and transferred to nitrocellulose filters (Sartorius) by the method of Southern.²⁰ Filters were prehybridized and subsequently hybridized with a nick-translated ³²P-probe according to standard methods.²¹

The immunoglobulin gene probes, kindly provided by Dr P. Leder (Harvard Medical School), were human genomic DNA fragments specific for the κ constant region Hu κC–2.5-kB Ri-Ri,²² the λ constant region Hu λC2–5-kB (kilobase) *Hind*III,²³ and the heavy-chain joining region Hu J_H–*Bam* *Hind*III,²⁴ all subcloned in

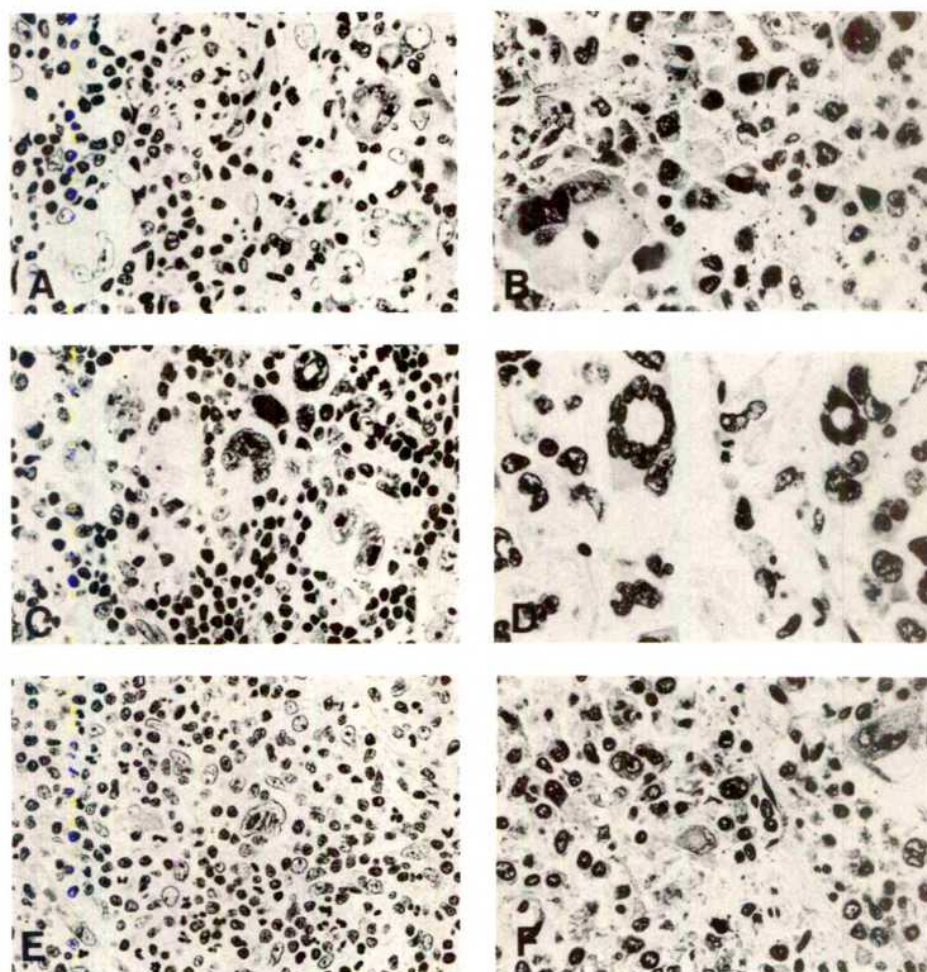


Fig 1. H&E-stained tissue sections of cases 3, 4, and 5. Figure 1A shows involved spleen and 1B involved lung tissue at autopsy of case 3. Figure 1C shows the diagnostic lymph node and 1D the involved liver tissue at autopsy of case 4. Figure 1E shows the diagnostic lymph node and 1F the involved lung tissue at autopsy of patient 5. In all tissues typical Reed-Sternberg cells can be identified, and in Figs 1B, D, and F, Reed-Sternberg cells and their variants predominate. (Original magnification ×350.)

pBR322. The probe for the T cell receptor α chain was pY1.4 Jurkat cDNA containing V, D, J, and C sequences and cloned into the *EcoRI* site of pUc8.²⁵ The probe for the T cell receptor β chain was a 770-bp (base pairs) Jurkat cDNA containing J and C sequences and cloned into the *PstI* site of pBR322.^{17,26} Both probes were provided by Dr Tak W. Mak (Ontario Cancer Institute). After hybridization, the filters were washed according to the procedure of Maniatis et al²¹ and exposed to Kodak XAR5 film with intensifying screens at -80°C for various periods (one to five days).

Immunohistological procedures. Immunohistological stainings were performed according to standard procedures.²⁷ Anti-B cell antibodies B1, B2, and B4, anti-T cell antibodies OKT11 and OKT3, anti-Reed-Sternberg cell reagents Ki-1 and Leu M1, anti-transferrin receptor T9, anti-HLA-DR antibody OKI1, and anti-interleukin 2 receptor antibody Tac were used (Table 2). Five-micrometer frozen sections were fixed in acetone, incubated with optimal dilutions of the monoclonal antibodies for 30 minutes, washed in phosphate-buffered saline (PBS) for five minutes, and subsequently incubated with horseradish peroxidase-conjugated rabbit antimouse Ig antibodies (Dakopatts, Copenhagen) diluted 1:20 for 15 minutes. After another wash for five minutes, the peroxidase activity was demonstrated by staining with 3-amino-9-ethylcarbazole and H_2O_2 for ten minutes, which resulted in a red reaction product. The sections were counterstained with hemalum to identify the Reed-Sternberg cells.

RESULTS

The histological findings in the diagnostic lymph node sections and in the involved liver and lung tissues at autopsy of patients 3, 4, and 5 are illustrated in Fig 1. Reed-Sternberg cells and their variants were the predominating cell type in the selected tissues.

The results of the immunoglobulin gene analyses in the patients with clonal rearrangements are shown in Figs 2 to 4 and summarized in Table 3. The tissues of patients 6 to 11 had germ line patterns with all probes used. All experiments were repeated with constant results. The DNA of patient 2 was also treated with double amounts of enzymes to exclude the presence of undigested DNA. Figure 2 shows the results of the hybridization with the J_H probe after enzymatic digestion with *Bam*HI. In the placental controls the probe hybridized with a 19-kB fragment, which might thus be

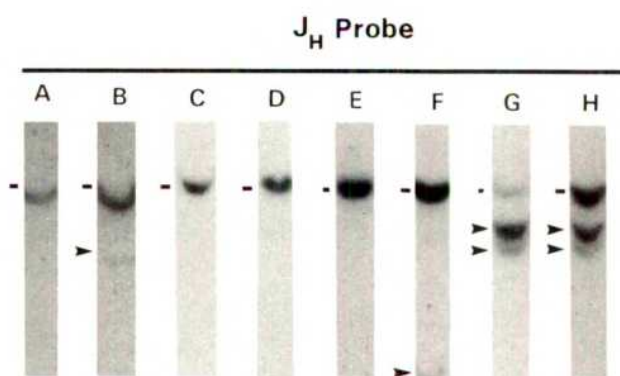


Fig 2. Autoradiographs of hybridization with J_H probe. Germ lines are indicated by dashes. Rearranged bands, indicated by arrowheads, are present in lanes B, G, and H. Results are shown for digestion with *Bam*HI, except for lane F, which was *Eco*RI digested.

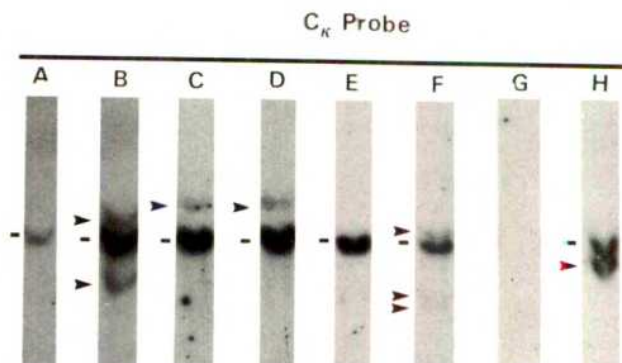


Fig 3. Autoradiographs of hybridization with κC probe. Germ lines are indicated by dashes. Rearranged bands, indicated by arrowheads, are present in lanes B, C, D, F, and H. In lane G the κC genes appear to be deleted. Results are shown for digestion with *Bam*HI.

considered to represent a germ line pattern. Rearranged bands were detected in lane B (lymph node patient 1), lane G (liver patient 4), and lane H (lung patient 5), and a germ line pattern was seen in lanes A, C, D, E, and F (placenta and patients 2 and 3). In some lanes an additional faint band can be observed below the germ line band. This band is also present in control placentas at longer exposure times. Hybridization with the J_H probe after enzymatic digestion with *Eco*RI gave rearrangements in the same samples as with *Bam*HI and, in addition, two rearranged bands in the lung tissue of patient 3. The rearrangements in lanes G and H, which appear similar after *Bam*HI enzymatic digestion, are clearly different from each other when *Eco*RI digestion is performed. Figure 3 shows the results obtained with the κC probe. Hybridizing 12-kB DNA fragments representing the germ line pattern were found in the placental controls. Rearranged bands were detected in lanes B (lymph node patient 1), F (lung patient 3), and H (lung patient 5), whereas the κC gene appears to be deleted in lane G (liver patient 4) where only a very faint germ line resulting from some normal admixed cells can be seen. Lanes C and D (lymph node and spleen of patient 2) showed faint rearranged bands, not present in normal liver tissue of this patient and, as mentioned previously, still present after

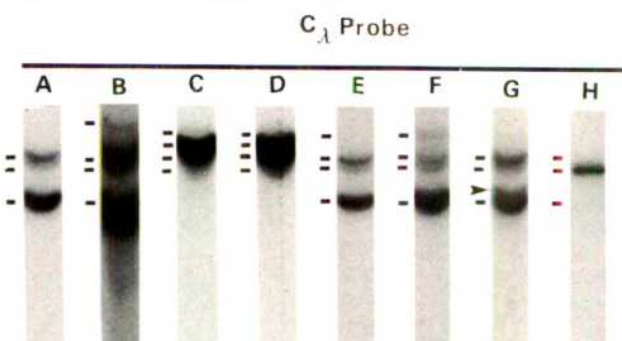


Fig 4. Autoradiographs of hybridization with $\lambda C2$ probe. Germ lines are indicated by dashes, which demonstrate the restriction fragment polymorphism of the λ gene locus. A rearranged band (indicated by an arrowhead) is present in lane G. Results are shown for digestion with *Eco*RI.

Table 3. Summary of Results in Tissues With Clonal Rearrangements of Ig Genes

Tissue	J _H	κC	λC2
Lymph node case 1	Rearranged	Rearranged	Germ line
Lymph node case 2	Germ line	Rearranged (faint)	Germ line
Spleen case 2	Germ line	Rearranged (faint)	Germ line
Spleen case 3	Germ line	Germ line	Germ line
Lung case 3	Rearranged (EcoRI)	Rearranged	Germ line
Liver case 4	Rearranged	Deleted	Rearranged
Lung case 5	Rearranged	Rearranged	Germ line

digestion with double amounts of enzyme. A germ line pattern was seen in lanes A (placenta) and E (spleen patient 3). Figure 4 shows the results obtained with the λC2 probe. Three different germ line patterns of hybridization with this probe were found in the placental controls. These patterns consisted of three fragments at 16 kB, 14 kB, and 8 kB; four fragments at 18 kB, 16 kB, 14 kB, and 8 kB; and three fragments at 18 kB, 16 kB, and 14 kB respectively. In addition, in some lanes faint bands were observed at 21- and at 5-kB positions. Rearranged bands were only detected in lane G (liver patient 5).

No rearrangements were found by using the TCR α probe, neither with *Bam*HI-digested DNA nor with *Eco*RI-digested DNA. Rearranged bands were also not detected in *Bam*HI-, *Eco*RI-, and *Hind*III-digested DNA by using the TCR β probe.

The results of the immunoperoxidase staining reactions in the cases with Ig gene rearrangements are summarized in Table 2. In all cases reactivity with anti-Reed-Sternberg cell antibody Ki-1 was observed. Leu M1 was reactive with all but one case (patient 4), generally with paranuclear cytoplasmic and membrane staining. B1 and B4 were reactive with the Reed-Sternberg cells in tissue sections of three patients, with variable proportions of positive cells and variable staining intensity. In one case (patient 3), several typical Reed-Sternberg cells showed membrane and cytoplasmic reactivity with B2 antibody. Generally, no reactivity of Reed-Sternberg cells was observed with anti-E rosette receptor antibody OKT11 and anti-T cell receptor antibody OKT3. However, in case 1 a few Reed-Sternberg cells with clearly positive OKT11 and OKT3 staining were observed, although the vast majority of Reed-Sternberg cells in this case were B1-positive. Staining with anti-Tac showed Reed-

Sternberg cells with cytoplasmic and also membrane staining of variable intensity (Fig 5). All Reed-Sternberg cells were strongly reactive with anti-transferrin receptor antibody OKT9 and anti-HLA-DR antibody OKI1. Cases 6 to 11 without detectable Ig gene rearrangements had similar immunophenotypes of the Reed-Sternberg cells as the positive cases.

DISCUSSION

The findings indicate the presence of clonal populations of cells possessing uniform rearrangements of immunoglobulin genes and the absence of clonal cell populations with rearrangements of T cell receptor genes. Similar results were obtained by Weiss et al in a number of cases with the recurrent nodular sclerosis type of Hodgkin's disease.³⁶ For an interpretation of the results, it should be kept in mind that Reed-Sternberg cells and their variants generally constitute only a minority of the cell population in tissue involved by Hodgkin's disease, the majority being T lymphocytes, macrophages, and eosinophils. Therefore, possible DNA rearrangements in minor clonal populations of Reed-Sternberg cells may stay below the threshold of sensitivity of the blotting technique. This may explain why in several lymph node samples only germ line configurations of the immunoglobulin and T cell receptor genes were observed, whereas in patients 1 and 2 only very faint rearranged bands were present. In addition, the possibility cannot be excluded that there are multiple clones of Reed-Sternberg cells in early Hodgkin's disease, each remaining below the threshold of sensitivity. The tissues obtained at autopsy showed a tumorous infiltration by Reed-Sternberg cells and only a minor admixture of other cell types, which explains the faintness or absence of germ lines in these samples. These tissues are representative for late stages of Hodgkin's disease when expansion of clonal populations may have occurred.

An unexpected finding, because it is not in agreement with the supposed sequence of heavy- and light-chain gene rearrangements, is the presence of a rearranged κ band in the lymph node and spleen of patient 2 without detectable heavy-chain rearrangement. A similar situation was observed in a patient with a lymphoproliferative disorder after cardiac transplantation.³⁷

However, since the sensitivity of the light-chain probes appears to be ten to 20 times higher than that of the J_H probe,³⁸ the rearranged population may have been too small to be detected in the J_H analysis. Alternatively, deletion of rearranged heavy-chain genes may have occurred. The faint



Fig 5. Immunohistological stainings of frozen tissue sections of the lymph node of patient 2 that demonstrate positive staining of Reed-Sternberg cells with Leu M1 (A), Tac (B), and Ki-1 (C). (Original magnification $\times 350$.)

extra 12-kB band observed in some lanes after hybridization with the J_H probe is also seen in all placental controls after longer exposure periods and has been described previously.³⁷ The faint extra 5-kB band observed in some lanes after hybridization with the $\lambda C2$ probe has previously been described as a $C\lambda$ pseudogene.¹⁹ The 21-kB band was found as a relatively unusual polymorphism by Hieter et al.²³ The importance of using different restriction enzymes (*Bam*HI and *Eco*RI) for DNA digestion and subsequent analysis with the J_H probe is exemplified by the absence of rearranged bands in patient 3 after *Bam*HI digestion and the presence of rearranged bands after *Eco*RI digestion. In addition, the rearrangements in cases 4 and 5, which appeared similar after *Bam*HI digestion, could be shown to be clearly different after *Eco*RI digestion.

The results of our study do not indicate which cell population within the tissue samples contained the clonal rearrangements. The presence of heavy-chain rearrangements has also been noted in myeloid and T cell neoplasms.^{39,40} So far light-chain rearrangements have been found exclusively in B cell neoplasms. Therefore it is likely that the rearranged immunoglobulin bands represent clonal B cell populations. The next question is whether these clonal B cell populations could be the Reed-Sternberg cells and their variants. Arguments in favor of this are the finding that clonal populations are found exclusively in cases with numerous Reed-Sternberg cells and not in cases with a predominance of small T or B lymphocytes and also that Reed-Sternberg cells in some cases express pan-B cell markers. However, in other cases Reed-Sternberg cells expressed no such B cell markers. Similar results were obtained in other studies.^{11,12} An alternative explanation could be the presence of a monoclonal population of B lymphocytes, benign or malignant, that could not be distinguished morphologically. This cannot be excluded in some of the cases in which significant numbers of

scattered B lymphocytes and reactive follicles are present. The absence of monoclonal immunoglobulin in the immunohistological stainings pleads against this possibility in our cases. Nevertheless, during this study we did exclude one case that had a composite lymphoma consisting of Hodgkin's disease and a lymphocytic lymphoma with plasmacytoid differentiation since the lymphocytes in this case clearly had monoclonal heavy- and light-chain immunoglobulin staining. In the autopsy cases the number of B lymphocytes as detected with antiimmunoglobulin reagents and pan-B cell antibodies was negligible, whereas the vast majority of the cells could be identified both morphologically and immunohistologically as Reed-Sternberg cells and variants. These arguments suggest that the clonal immunoglobulin gene rearrangements derive from Reed-Sternberg cells. This implies that the Reed-Sternberg cells in these cases are of B cell origin. However, to prove that Reed-Sternberg cells are B cells, it will be necessary to isolate these cells and demonstrate Ig gene rearrangements and Ig messenger RNA and to induce these cells to produce immunoglobulin since this appears to be the only absolute proof for the B cell character of cells. All other markers, including the gene rearrangements, can only provide strong arguments for the lineage of neoplastic cells.

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Growth Factor Requirements of Childhood Acute Leukemia: Establishment of GM-CSF-Dependent Cell Lines

By Beverly Lange, Mauro Valtieri, Daniela Santoli, Daniele Caracciolo, Fulvio Mavilio, Ingrid Gemperlein, Constance Griffin, Beverly Emanuel, Janet Finan, Peter Nowell, and Giovanni Rovera

Eight permanent cell lines were established from cells of 50 consecutive patients with childhood acute leukemia. Three cell lines required growth factor-containing conditioned media. Analysis using blocking antisera and recombinant granulocytic macrophage (GM) colony-stimulating factor (CSF) identified GM-CSF as a growth factor required to establish the latter three cell lines and necessary for their continuous proliferation in chemically defined medium. Two of the GM-CSF-dependent cell lines were derived from patients with undifferentiated T- and a biphenotypic B-myelomonocytic leukemia, which suggests that GM-CSF might maintain proliferation of leukemias originating from

immature progenitor cells. Cytogenetic analysis indicated that all established leukemic cell lines were aneuploid, with six lines containing chromosomal alterations related to those observed in the leukemic cells of the patient. Two patients did not have an abnormal clone identified in the marrow but did yield an aneuploid cell line. These studies indicate that GM-CSF-dependent leukemic cell lines can be established in a fraction of childhood leukemia. These cell lines lend themselves to studies aimed at the evaluation in vitro of the role of growth factors in controlling proliferation and differentiation of leukemic cells.

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HUMAN LEUKEMIC CELLS usually fail to proliferate autonomously in vitro.¹⁻³ The dependence of human leukemic cell proliferation on specific hematopoietic growth factors was first demonstrated in well-differentiated or partially differentiated human T leukemia cells that grow in media supplemented with interleukin-2 (IL2).⁴ However, highly undifferentiated leukemic T cells do not respond to IL2. The growth factors required to sustain proliferation in vitro of human acute myeloblastic leukemia (AML) and pre-B leukemia cells are not yet well defined. A small fraction of AML cells can form colonies in semisolid media supplemented with conditioned media (CM) containing colony-stimulating activity for normal myeloid cells.^{1,5,6} Recent work using recombinant granulocytic macrophage colony-stimulating factor (GM-CSF) has indicated that this growth factor can efficiently replace CM to sustain limited proliferation of leukemic cells.^{7,8} However, the leukemic colonies usually undergo terminal differentiation, and subculturing is not possible for more than two or three passages. The long-term suspension culture system for normal myeloid and erythroid precursor cells⁹ has proved to be inadequate for human leukemia cells since it tends to select for growth of normal rather than malignant clones.¹⁰ Long-term culture of AML using CM from phytohemagglutinin-stimulated lymphocytes¹¹ or from a bladder carcinoma cell¹² has been successful in sustaining the growth of leukemic cells in

suspension for several weeks, but growth factor-dependent AML lines have not been reported.

We have cultured 50 consecutive cases of acute childhood leukemia in standard media and serum and in the presence of medium conditioned by three cell lines (Mo-T, JLB-1, and 5637) known to release into the medium a variety of hematopoietic growth factors.¹³⁻¹⁹ These cultures were used to determine (a) the frequency of leukemias able to continuously proliferate in the presence or absence of CM and (b) the specific factors responsible for such proliferation.

MATERIALS AND METHODS

Leukemic cells. Blast cells were obtained from the marrow or peripheral blood of 50 consecutive children with acute leukemia at diagnosis or at the time of relapse. Cases were classified according to the French-American-British (FAB) system.²⁰ Cases were also classified by risk categories according to Children's Cancer Study Group criteria.²¹ The described work was approved by the Human Subject Committee of Children's Hospital of Philadelphia, and informed consent was obtained in every case.

Long-term culture conditions and preparation of CM. Long-term cultures were established by using minor modifications of the procedures described.^{8,11} Low-density ($d = 1.077$) mononuclear cells were separated on a Ficoll-Hypaque gradient,²² washed twice in phosphate-buffered saline, and resuspended at 10^6 cells/mL in Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) and 20% fetal bovine serum (FBS) (Hyclone, Logan, Utah) alone or with 10% filtered supernatant fluid of one of three cell lines: Mo-T,¹³ JLB-1,¹⁵ or 5637.¹⁶ For preparation of CM cells of these lines were grown to 70% to 80% confluence and placed in chemically defined medium (IMDM supplemented with insulin [$10 \mu\text{g/mL}$] and transferrin [$10 \mu\text{g/mL}$]).²³ Serum-free CM was collected five days later. Leukemic cells were placed in 24-well cluster dishes (Costar 3424, Costar, Cambridge, MA) in 5% CO_2 at 37°C . Cultures were adjusted to 10^6 cells/mL and refed at weekly intervals.

Short-term proliferative assays and clonogenic assays. To determine the proliferative response of the leukemic cell lines in vitro in the presence of CM and growth factors, 5×10^4 cells were plated in microwells and after three days in culture labeled with $1 \mu\text{Ci/mL}$ of ^3H -thymidine for 18 hours. After harvesting the cells, radioactivity incorporated into the DNA was determined by liquid scintillation counting. The ability of leukemic cells to form colonies in the presence of growth factors (CFU-L) was determined by using a modification of the method of Buick et al²⁴ and plating 1×10^4 cells

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in 1% ultralow-gelling agarose (FMC, Rockland, ME) containing 10% CM and 20% FBS and scoring the resulting colonies after seven to ten days. Clones were obtained by picking up the colonies with a Pasteur pipette and expanding them in liquid cultures.

Phenotypic characterization of long-term cultures and cell lines. Cells were collected and concentrated on slides by cytocentrifugation (Shandon Southern Products, Sewickley, PA) and stained for PAS, Sudan black, myeloperoxidase, nonspecific esterase, chloroacetate esterase, and acid phosphatase using established methods.^{25,26}

Indirect immunofluorescence assays were performed as described²⁷ by using an Ortho Cytofluorograf 50HH (Ortho Diagnostic Systems, Inc, Westwood, MA). Epstein-Barr nuclear antigen (EBNA) fluorescence assay was carried out on fixed slides as described by Reedman and Klein.²⁸

Karyotypic analysis. Karyotypic analysis of overnight cultures and direct preparations of fresh samples obtained from patients, of established cell lines, and of cultures proliferating for longer than 8 weeks was performed by standard techniques.²⁹

DNA preparation, Southern and Northern blot analysis. High-molecular weight DNA was isolated from tissue culture cells by using sodium dodecyl sulfate-proteinase K digestion.³⁰ After restriction enzyme digestion, the DNA was blotted to nylon membranes (Zetabind AMF Cuno, Meriden, CT) and covalently bound to the membrane by UV irradiation.³¹ Blots were prehybridized and hybridized with oligo-labeled probes³² and washed according to established procedures. Blots were exposed to Kodak XAR film for 24 hours at -70°C .

Cytoplasmic RNA was prepared by following the procedure of Chirgwin et al.³³ Five to 10 μg were electrophoresed on formaldehyde-containing agarose gels.³⁴ The blotting and hybridization procedures were similar to those used for Southern blots.

Reagents and probes. Recombinant human (rh) GM-CSF, G-CSF, and anti-GM-CSF sheep antiserum were gifts of Dr Steven Clark (Genetics Institute, Boston). A plasmid containing the 3.3-kb germline *EcoRI-HindIII* μ fragment was a gift of P. Leder (Harvard University, Boston). Plasmids containing the 0.30-kb *MboII-HindI* fragment of the *Ck* gene and an 8.0-kb *EcoRI* fragment containing the *CX2* and *CX3* genes were gifts of T.H. Rabbitts (MRC Laboratory of Molecular Biology, Cambridge, UK).³⁵ The T cell receptor β -chain constant-region probe was the *HincII* 3' fragment of the human YTJ-2 cDNA clone and was provided by Dr T.W. Mak (University of Toronto).³⁶ A plasmid (pH 13) containing the 5' end of the human T-lymphotropic virus II (HTLV II)³⁷ was a gift of Dr D.W. Golde (UCLA, Los Angeles). A plasmid (B95-8 *EcoRI* C fragment) containing a 9.5-kb fragment of the Epstein-Barr virus (EBV) genome³⁸ was obtained from Dr N. Fraser (The Wistar Institute, Philadelphia).

Monoclonal antibodies T11, T10, T3, T6, B4, and M1 were purchased from Ortho Diagnostic Systems, Inc (Westwood, MA); Leu1, LeuM2 and LeuM3 from Becton Dickinson (Mountain View, CA); J5 and Ia from New England Nuclear (Boston); My3, -4, -7, and -9 from Coulter Immunology (Hialeah, FL); and 3A1 from the American Type Culture Collection (Rockville, MD). Monoclonal antibodies R1B19, S3.13, S4.7, and S16.144 were characterized in this laboratory and have been described.³⁹

RESULTS

Tables 1 to 3 summarize the characteristics of the 50 acute childhood leukemias included in this study. Thirty of these cases were classified as pre-B (Table 1), seven as T (Table 2), and 13 as AML leukemias (Table 3). Thirty-four cases were from untreated, newly diagnosed patients, 15 were in relapse, and one (patient 49) was a second malignant neoplasm in a

Table 1. Cases of Childhood Leukemia: Pre-B ALL

No.	Age	Sex	Blasts (%)	FAB	Risk	Status	Karyotype
1	2	M	99	L1	M	U	46, XY, del(12)(p11)
2	2	F	99	L1	M	U	No mitoses
3	2	F	98	L1	M	U	No mitoses
4	3	F	46	BO	—	R	46, XX, +1q—, -17
5	10	F	99	L2	H	U	46, XX
6	8	M	99	BO	—	R	No mitoses
7	12	M	28	L2	M	R	47, XY, t(1;19)(q24;p13), 6q—, t(12;7)(q21;7), +mar
8	6	F	99	L1	M	U	46, XX, t(20;7)(q11;7), t(22;7)(q11;7)
9	4	F	95	L1	M	U	46, XX
10	5	M	99	L1	L	U	46, XY
11	6	F	99	L1	L	U	46, XX
12	1 (mo)	F	99	L1	H	U	46, XX, t(4;11)(q21;q23) 47, XX, t(4;11)(q21;q23), +C
13	6 (mo)	F	44	L1	H	U	46, XX;
14	2	F	99	L1	H	U	46, XX
15	16	M	81	L1	M	U	No mitoses
16	21	F	99	L1	M	R	No mitoses
17	6	F	99	L1	M	U	46, XX
18	4	M	98	L1	M	U	47, XY, Gq—(?Ph), +mar
19	3	F	99	L1	L	U	No mitoses
20	14	F	95	L1	M	U	46, XX
21	16	F	96	L1	M	U	No mitoses
22	9	M	95	L1	M	U	45, XX, +A, -B or C, +D or E size, -2G, 2q+
23	11	F	98	L1	M	U	46, XY
24	11	F	49	L1	M	U	No mitoses
25	3	M	99	L1	M	U	46, XY/hypotetraploid (84)
26	10	F	99	L1	M	R	46, XX, del(6)(q23), del(17)(p11), t(12;7)(p13;7), der(19) t(1;19)(q23;p13), +der(1) t(1;9)(p11;q11)
27	12	F	99	L2	M	R	46, XX, r(1), -21, +mar
28	5	M	63	L1	M	U	52-55, XY, +5C, +2E, +2G
29	6	F	99	L1	M	R	26, -X, -1, -2, -3, -4, -5, -7C, -3E, -2F, -G
30	9	M	99	L1	M	U	46, XY

NOTE: Risk refers to the risk of relapse according to the Children Cancer Study Group criteria.

Abbreviations: L, low; M, moderate; H, high; R, relapse; U, untreated; mar, marker chromosome.

Table 2. Cases of Childhood Leukemia: T ALL

No.	Age	Sex	Blasts (%)	FAB	Risk	Status	Karyotype
31	4	M	99	L1	H	R	46, XY
32	5	M	99	L1	H	U	46, XY
33	8	F	95	L2	H	U	46, XX
34	9	M	99	L1	H	U	46, XY
35	9	M	99	L1	H	R	46, XY, t(8;14)(q24;q11) inv(9)
36	18	M	96	B1	H	R	45, X, -Y
37	4	M	99	L1	H	U	46, XY, 1p+, 14q—

Table 3. Cases of Childhood Leukemia: AML

No.	Age	Sex	Blasts (%)	FAB	Risk	Status	Karyotype
38	13	F	99	M5	H	R	46, XX, i(17q)
39	6	M	99	M2	H	U	46, XY, t(9;15)
40	13	F	99	M3	H	R	47, XX, t(15;17)(q22;q12)+8 48, XX, t(15;17), +8, +mar, 46, XX, t(15;17)(q22;q12)
41	2	M	99	M5	H	U	46, XY
42	1	M	30	M4	H	U	47, XY, -7, +21, +i(17q) (Down's)
43	12	F	98	M3	H	U	46, XX, t(15;17)(q22;q12-21)
44	14	F	55	M4	H	R	47, XX, +21, t(11;20)(p15;q11)
45	14	F	99	M6	H	U	46, X0 (constitutional)
46	12	M	99	M4	H	R	55, XYY, +4, +6, +7, +8, +9, +14, +19, +21 52, XY, +del(1)(p31), +6, +8, +8, t(12;7)(p13;?), +19, +21
47	18	M	50	M2	H	R	46, XY
48	3	F	95	M5	H	R	46, XX, -2, -2, -8, -17, 9q-, +4mar
49	11(mo)	M	95	M5	H	R	46, XY, t(1q32;?), t(2p21;?)(2q37;?), t(3q25;?), t(7q32-35;?), t(11q23-25;?)
50	10	M	95	M5	H	U	46, XY, t(4;11)(q21;q23)

boy who had a marrow transplant for neuroblastoma. Among patients with pre-B acute lymphocytic leukemia (ALL), 3 were originally in the low-risk category, 21 were moderate risk, 4 were high risk, and 2 were not classified insofar as they had Sudan black-positive blasts with lymphoid morphology and a pre-B immunologic phenotype. Both had failed AML therapy. Among the T cell leukemias, six of eight were high risk, one was moderate risk, and one was a biphenotypic T cell monoblast with a predominance of T cell lymphoblasts. Cells from two patients had L2 morphology and 5 had L1 morphology. All AML patients are at high risk of relapse.

In the original chromosome studies on these 50 patients, a karyotypically abnormal clone replacing all or part of the bone marrow was found in 24 patients; only normal metaphases were found in 18 patients. Results were inconclusive in eight cases.

The percentage of blast cells in the marrow used for these studies ranged from more than 90% in the majority of cases to 28% in one case of pre-B ALL.

Establishment of cell lines. Of the 50 cases, cell lines growing for more than 1 year in culture were established from ten cases. Two of these cell lines (cases 20 and 39) consisted of EBV-transformed cells, as demonstrated by EBNA immunofluorescence and by Southern blotting with an EBV specific probe, and were discarded. The eight remaining cases were derived from three pre-B, two T, and two AML, one of which was eventually identified as a

Table 4. Genotype of Established Human Leukemia Cell Lines

Cell Lines	Recombinant Probes			
	J μ	K	λ	T
ALL-1	R/D	R/R	G	G
ALL-2	R/R	D/D	G	G
ALL-202	R/G	G	G	G
TALL-1	G	G	G	R/R
TALL-101	G	G	G	R/G
AML-193	G	G	G	G
AML-1	G	G	G	G
MV4-11	G	R/G	G	G

The double letters indicate the fate of the two different alleles.

Abbreviations: R, rearranged; D, deleted; G, germ line.

biphenotypic (B-myeloid) leukemia (see Table 4). None of the three pre-B lines required CM for growth. The cell line derived from case 35 (TALL-101) required CM from JLB-1 cells but grew almost as well in Mo-T and 5637 CM. The T cell line TALL-1 (from case 31) grew without CM. Among the AML lines, line AML-1 (from case 46) grew spontaneously, and AML-193 (from case 38) and MV4-11 (from case 50) grew equally well in Mo-T, JLB-1, or 5637 CM. In all cases, proliferation began during the first week in culture and continued at approximately the same rate thereafter.

Identification of GM-CSF as the growth factor required for maintenance and establishment of some cell lines. Multiple factors are present in the CM of HTLV-transformed T cells,¹³ and the media from the Mo-T cell line

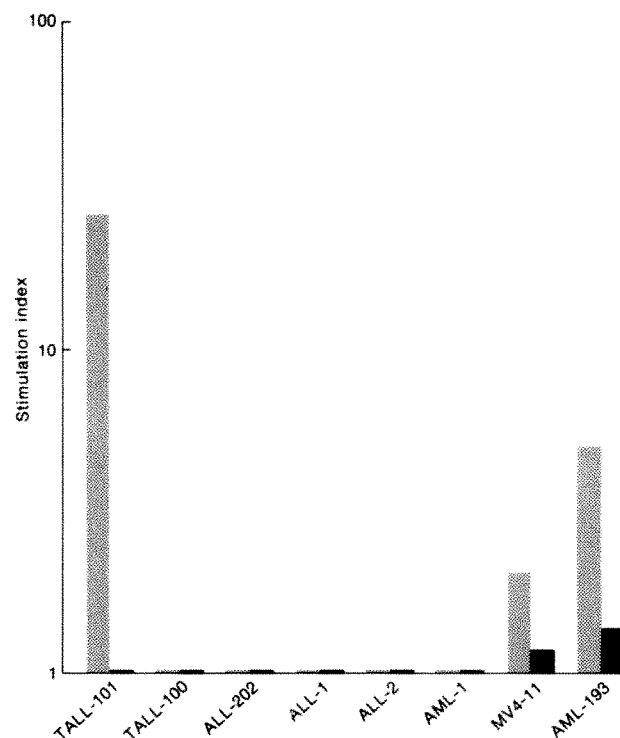


Fig 1. Effect of G- and GM-CSF on thymidine incorporation of eight cell lines. Control cultures were maintained in IMDM supplemented with 20% FBS. Ordinate: stimulation index = cpm experimental/cpm of control cultures.

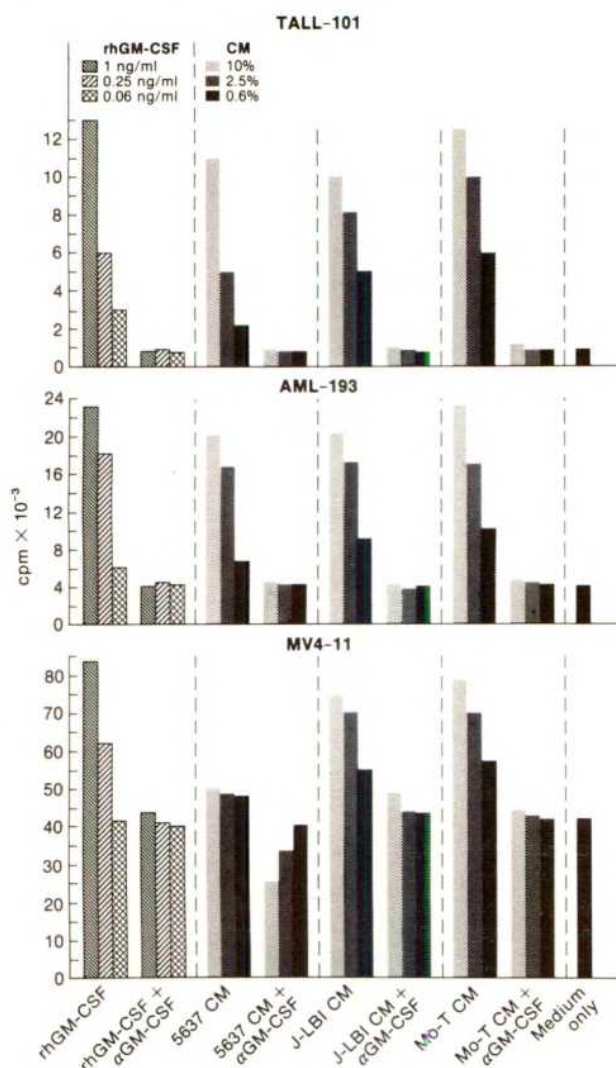


Fig 2. Effect of blocking antiserum against GM-CSF on three cell lines growing in CM.

have been used as the source for purification of GM-CSF.¹⁴ Three factors have been identified in the CM of 5637, ie, G-CSF,¹⁷ GM-CSF,¹⁸ and hemopoietin L,¹⁹ a factor shown to render cells competent for the effect of M-CSF. Since the three CM had approximately the same biological activity in stimulating leukemic cell cultures and all three cell lines produce GM-CSF, we investigated the role of GM-CSF in the establishment of the cell lines.

The five EBV-negative cell lines that established spontaneously and the three cell lines that required CM were screened for their proliferative response to recombinant growth factors. Thymidine incorporation was measured after addition of GM-CSF or G-CSF at varying concentrations to the cell lines growing in IMDM supplemented with 10% serum. Only the three lines that required CM for establishment proliferated in response to recombinant GM-CSF treatment (Fig 1). The addition of GM-CSF-specific blocking antiserum to cells growing in the media supplemented with the three CM reduced ³H-thymidine incorporation to levels observed in the absence of GM-CSF (Fig 2), thereby

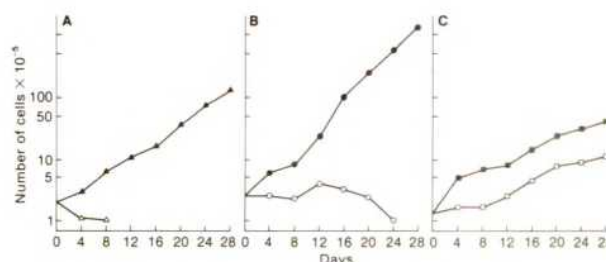


Fig 3. Growth curve of TALL-101 (panel A), AML-193 (panel B), and MV4-11 (panel C) cells grown in chemically defined medium alone or supplemented with rhGM-CSF (10 ng/mL).

indicating that GM-CSF accounts for the stimulatory activity in the different CM.

To determine whether GM-CSF was sufficient to support the continuous proliferation of the cells in the absence of other factors in serum, the three cell lines were transferred to chemically defined medium and their growth in the presence or in the absence of GM-CSF compared (Fig 3). Two of the cell lines (AML-193 and TALL-101) clearly maintained their requirement for GM-CSF. MV4-11 cells were able to grow even in the absence of GM-CSF, but the proliferation rate was approximately 50% less than that of the same cells growing in GM-CSF. The frozen leukemic cells of the patient from which the MV4-11 cell line was derived were cloned in a CFU-L assay using medium only or medium supplemented with GM-CSF (10 ng/mL) (see Table 5). Colonies could be obtained only in plates containing GM-CSF. When the colonies were individually picked and replated, only a small fraction (4%) grew in culture, and again, they rapidly became independent of the requirement for GM-CSF.

To rule out autocrine production of growth factors as the mechanism controlling proliferation of the cell lines, the RNA of the eight cell lines was probed by Northern blot analysis using a GM-CSF and a G-CSF probe. No specific mRNA for such a growth factor could be detected (not shown).

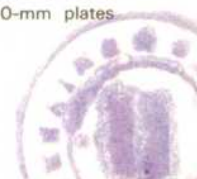
Genotypic characterization. To determine whether lineage-specific gene rearrangement was present in the eight cell lines, their DNA was digested with several restriction enzymes and hybridized to recombinant DNA probes specific for the IgM heavy chain, κ and λ light chains loci, and for the T β cell receptor locus. Representative patterns of

Table 5. Analysis of Reestablishment of MV4-11 Cell Line by Using rhGM-CSF

	Number of Colonies on Day 14*	Clones Able to Generate Secondary Colonies† (%)	Growth in Chemically Defined Medium
Minus GM-CSF	0	—	—
Plus rhGM-CSF (10 ng/mL)	850	4	+

*Values indicate the mean colony number on triplicate plates in a CFU-L assay. A total of 10⁴ cells were seeded in 30-mm plates containing 1% low-melting agarose.

†A total of 25 individual colonies were examined.



rearranged genes are shown in Fig 4. The three cell lines with a pre-B phenotype (ALL-1, ALL-2, and ALL-202) also had a pre-B genotype with rearrangement of $J\mu$ in the case of ALL-202, rearrangement of $J\mu$ and κ in the case of ALL-1 and rearrangement of $J\mu$ and deletion of both κ loci in the case of ALL-2 (Fig 4A and B). Of the two cell lines derived from patients with T leukemias, both had a T cell genotype showing a rearranged $T\beta$ gene. TALL-1 cells had a rearrangement in the $T\beta 1$ gene (Fig 4C) and actively transcribe $T\beta$ RNA (not shown), whereas TALL-101 showed a nonproductive rearrangement in the $T\beta 2$ locus.⁴⁰ Myeloid cell lines (AML-193, AML-1) showed a germ line configuration for all genes tested. MV4-11 cells with a t(4;11) translocation and clinically diagnosed as an AML have a rearranged κ light chain but not $J\mu$ (Fig 4B). Table 4 summarizes the genotype of the cell lines. Since three cell lines (AML-193, TALL-101, and MV4-11) were established with the CM of HTLV II-transformed cells, Southern blots were done with an HTLV II-specific probe; no hybridization was detected.

Phenotypic characterization. Table 6 compares the phenotype of the cell lines with those of the patients' leukemic cells. The morphological, histochemical, and immunologic phenotype of the cell lines were usually but not always identical to those of the patients' blast population present in vivo. For example, in the case of TALL-101 (case 35), the T11 marker was lost, and we were unable to demonstrate the presence of T lineage-specific markers.

Karyotype characterization. Six of the cell lines showed abnormal karyotypes related to those of the corresponding leukemic cells of the patient (Tables 1 to 3 and 7). In two cases (5 and 31) the derived cell lines were aneuploid, but only normal metaphases were observed in the patient's bone marrow. Cells of line ALL-1 have either one or two Philadelphia chromosomes resulting from the typical t(9;22) translocation. ALL-2 appears to have most of a chromosome 1q translocated to 19p. ALL-202 is missing a C-group chromosome and contains a small marker chromosome of undefined

origin. Both of the T-ALL lines carry translocations involving 8q24, the region of the *c-myc* locus, and TALL-1 (as well as ALL-2) also has a 6q- chromosome. The AML-193 line has an isochromosome 17q, and AML-1 has extra copies of chromosome 8. MV4-11 has the translocation t(4;11)(q21;q23), typically observed in biphenotypic congenital leukemias.^{41,42}

DISCUSSION

The present studies were done to determine the frequency of childhood leukemias able to continuously proliferate in standard or conditioned culture medium and to define the factors in CM responsible for proliferation.

We examined the proliferative response in vitro of 50 consecutive cases of acute leukemia at diagnosis or relapse. Of 50 cases, 13 (26%) were maintained in culture for more than 2 months, and their derivation from the leukemic cells was documented by chromosomal analysis. Among these, eight cases (16%) gave rise to permanent cell lines. Of these eight cases, five grew in medium supplemented with FBS, with no delay in growth, and three cases required GM-CSF for the establishment of the cultures. In the latter three cases, the original diagnosis was acute monocytic leukemia (AML-193), undifferentiated (TALL-101), and myelomonocytic leukemia (MV4-11), which later was modified to a B-myeloid leukemia with a typical t(4;11) translocation.

When grown in chemically defined medium, AML-193 and TALL-101 cells continued to require GM-CSF for growth. The effect of GM-CSF on the growth of early T and MV4-11 biphenotypic cells supports the concept that this factor acts on immature cells of several lineages.⁴³ It was recently shown that rhGM-CSF is effective in generating colonies of AML,⁸ but attempts to establish lines from the growing colonies have not been reported. Recently, Young and Griffin have reported AML cell lines that require GM-CSF and endogenously produce GM-CSF.⁴⁴ Under the culture conditions used, we have been unable to detect

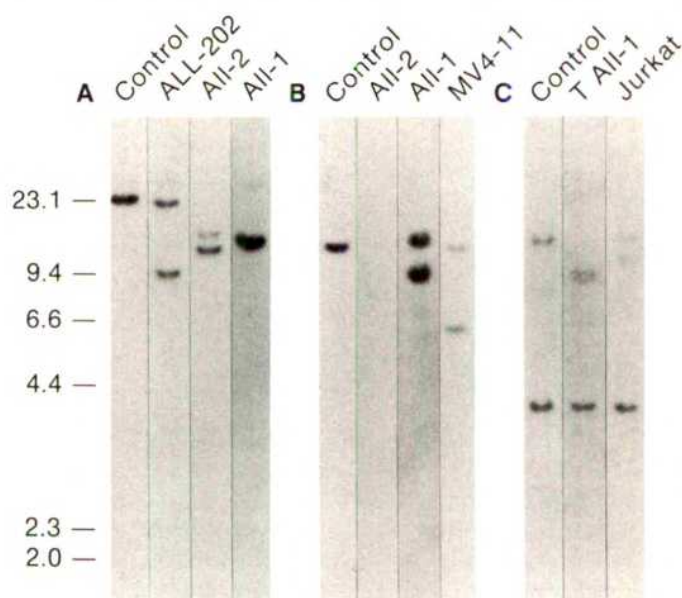


Fig 4. Southern blot analysis of DNA from eight leukemic cell lines. High-molecular weight DNA was digested with *EcoRI* (panels A and C) or *BamHI* (panel B) and hybridized with Ig $J\mu$ probe (A) and κ (B), specific genomic fragments, and to a full length cDNA for the β chain of T cell receptor (C). Migration of λ *HindIII* fragments is shown as size markers (Kb). The Jurkat cell line DNA is shown as an additional control for a rearranged $T\beta$ locus.

Table 6. Comparison of the Phenotype of the Leukemic Cells and of the Corresponding Established Cell Lines

	Histochemistry				Immunophenotype											
	PAS	PX	CAE	NSE	DR	CALLA	M1	M3	My4	My7	My9	S4-7	T11	T3	Leu1	3A1
Pre B ALL																
Case 88	+	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—
ALL-1	+	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—
Case 26	—	—	—	—	+	+	—	—	—	—	—	—	—	—	+	—
ALL-2	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—
Case 5	+	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—
ALL-202	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	—
T-ALL																
Case 31	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—
TALL-1	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+
Case 35	—	—	—	—	—	—	—	—	—	—	—	—	+	—	+	—
TALL-101	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—
ANLL																
Case 38	±	—	—	+	+	—	+	—	—	—	—	—	—	—	—	—
AML-193	±	—	—	±	+	—	+	—	—	+	—	+	—	—	—	—
Case 46	—	+	+	+	+	—	+	—	—	+	—	+	—	—	—	—
AML-1	—	—	+	+	+	—	+	+	+	+	+	+	—	—	—	+
Case 50	—	—	±	+	+	—	+	—	+	+	+	+	—	—	—	—
MV4-11	—	—	±	+	+	—	+	—	+	+	+	+	—	—	—	—

Abbreviations: PAS, periodic acid-schiff; PX, peroxidase; CAE, chloroacetate esterase; NSE, nonspecific esterase; P, acid phosphatase, +, >10% blasts positive, for monoclonal antibodies, + refers to >10% above control value; ANLL, acute nonlymphocytic leukemia.

production of GM-CSF by any of our cell lines either by Northern blot analysis or by bioassays of their conditioning media on human marrow cells in a colony-forming assay. Also, in the presence of GM-CSF, TALL-101 and AML-193 cells showed no density dependence within the range of cell densities (1 to 10×10^5) used in culture.

All of the established lines had an abnormal karyotype, and in several cases, this represented a further evolution of the chromosomal alterations originally observed in the patient. Four of the five lines with a lymphoid phenotype had karyotypic changes previously reported as occurring nonrandomly in lymphocytic neoplasms (ie, Philadelphia chromosome, translocations involving 8q24 and 19p13, a 6q chromosome). Similarly, the two myeloid lines had abnormalities commonly associated with nonlymphocytic leukemia (ie, $iso17q$, trisomy 8),⁴⁵ and the MV4-11 line has the $t(4;11)$ translocation that characterizes a subgroup of acute childhood leukemias.^{41,42} No diploid leukemic cell lines were established, and the question remains as to how often karyotypically normal metaphases from leukemic bone marrow represent the neoplastic population v residual nonneoplastic elements.⁴⁶ Interestingly, in our series, two patients who did

not have a chromosomally aberrant clone identified in the marrow did yield an aneuploid cell line.

From the relatively small number of cases in the present study, it is not possible to conclude whether cell lines with a specific chromosomal anomaly can be more readily established, either in the presence or the absence of growth factors. Current work is aimed at elucidating these relationships.

As previously discussed by Nara and McCulloch and Hoang and McCulloch,^{11,12} long-term cultures of leukemic cells may be more valuable than clonogenic assays in clinical studies. The relatively short clonogenic assays do not discriminate between cells with self-renewal ability and cells that proliferate and differentiate. The ability to maintain proliferating leukemic cells in culture should help determine the effects of long-term exposure to physiological or pharmacological agents that can potentially interfere with their growth in vivo. It may also permit the study of the development of resistance in the presence of relevant chemotherapeutic agents. All of the leukemic cell lines reported thus far are able to grow in medium supplemented with FBS, and no specific growth factor-dependent lines have been described. The GM-CSF-dependent lines TALL-101 and AML-193 and the GM-CSF-responsive cell line MV4-11 represent novel models to study in vitro the role of growth factors in supporting leukemic growth and to study the interaction of multiple growth factors on proliferation and differentiation of leukemic cells. Furthermore, the identification of factors that stimulate growth of leukemic cells would help to determine whether growth requirements of leukemic cells are different from those of normal myeloid progenitor cells. No spontaneous differentiation of the GM-CSF-dependent cells has been observed in culture. However, an increase of some lineage-specific markers was observed when TALL-101 were treated with interferon γ or tumor necrosis factor,⁴⁰ which

Table 7. Karyotype of Established Leukemic Cell Lines

No.	Cell Line	Karyotype
8	ALL-1	47, XX t(9;22)(q34;q11), + Ph
26	ALL-2	46, XX, -9, 6q-, 17p-, 12p+, der(19) t(1;19), + der(1) t(1;9)
5	ALL-202	46, XX, -C(79), + minute
31	TALL-1	46, XY, del(6)(q23), t(1;8)(q32;q24)
35	TALL-101	46, XY, t(8;14), (q24;q11), inv(9)
38	AML-193	46, XX, -17, +i(17q)
46	AML-1	51, XY, +1p-, +6, +8, +8, +19, 2p-, 12p+
50	MV4-11	48, XY, t(4;11)(q21;q23), +8, +19

suggests that these cells may be suitable for studies on the role of specific differentiating factors in the presence and in the absence of growth factors.

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Recombinant Human Tumor Necrosis Factor α Regulates *c-myc* Expression in HL-60 Cells at the Level of Transcription

By A. Tobler, D. Johnston, and H.P. Koeffler

Recombinant human tumor necrosis factor α (TNF α) effectively inhibits clonal growth of leukemic cells from patients and several cell lines, including the promyelocytic HL-60 cells. Decreased expression of the *c-myc* oncogene is linked to growth arrest and terminal cellular differentiation. The present study characterizes the effect of TNF α on the regulation of the *c-myc* gene in HL-60 cells. TNF α (100 U/mL) rapidly inhibited messenger RNA (mRNA) accumulation of *c-myc* with a 50% reduction in <one hour. Dose-response studies showed that a 50% reduction of *c-myc* mRNA occurred in the range of 15 U/mL. In vitro nuclear run-on experiments showed that this decrease of *c-myc*-mRNA accumulation was the result of a reduced rate of transcription of *c-myc* by TNF α . Further studies demon-

strated that TNF α did not post-transcriptionally alter levels of *c-myc* mRNA, and the inhibitory action of TNF α on *c-myc* expression in HL-60 cells did not depend on new protein synthesis. In the conditions of all the experiments, TNF α did not affect cell viability. By contrast, TNF α (500 U/mL) did not decrease mRNA levels of *c-myc* in an HL-60 variant cell line whose growth was not inhibited by TNF α ; also TNF α (500 U/mL) increased *c-myc*-mRNA levels in normal fibroblasts whose growth is known to be stimulated by TNF α . These findings, in concert with prior studies, show a close association between growth inhibition of HL-60 cells and decreased levels of mRNA coding for *c-myc*.

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RECOMBINANT human tumor necrosis factor α (TNF α) is a glycoprotein produced by myeloid cells. TNF α can potentially inhibit proliferation of leukemic cells from various human myeloid lines and from patients.¹ Part of the growth inhibitory action of TNF α on leukemic cells, including HL-60 promyelocytes, can be explained by induction of terminal differentiation.^{1,2} However, this cannot be the sole mechanism of growth inhibition because TNF α inhibits growth of cells from several myeloid lines that cannot be induced to differentiate by TNF α .¹ The gene product of *c-myc* is a nuclear protein³ linked to the control of normal cell proliferation and to neoplastic growth.^{4,7} The *c-myc* gene is amplified and transcribed at a high level in HL-60 cells.⁸⁻¹⁰ We show that TNF α rapidly and profoundly inhibits the expression of *c-myc* in HL-60 cells at the level of transcription, and this regulation occurs independently of new protein synthesis.

MATERIALS AND METHODS

Cell Culture

The promyelocytic leukemic cell line HL-60 was grown in alpha medium and 10% fetal calf serum (FCS) in a humidified atmosphere

with 7% CO₂. The TNF α (3.7×10^7 U/mg) was kept at 4°C and before use was diluted in alpha medium plus 10% FCS to the final concentrations. The TNF α -resistant HL-60 cell line was established by growing HL-60 cells in the presence of increasing concentrations of TNF α (1 to 50,000 U/mL) over a period of 6 months. Normal lung fibroblasts were obtained from a healthy volunteer, after informed consent was given, and were cultured in alpha medium and 10% FCS. The colony-forming assay in soft agar was performed as described.¹¹ Cell viability was determined in all experimental procedures by trypan blue exclusion.

RNA Isolation and RNA Blotting

Total RNA of HL-60 cells was extracted by the hot phenol method.¹² Cytoplasmic RNA of fibroblasts was isolated by phenol/chloroform extraction.¹³ RNA blotting was basically done as described.¹⁴ RNA samples were separated by a 1% agarose gel and transferred to a nylon-membrane filter. Blots were probed with [³²P]-oligolabeled (random primed) restriction fragments¹⁵ and hybridized for 16 to 24 hours at 42°C in 50% formamide, 2x SSC (1x = 150 mmol/L NaCl, 15 mmol/L sodium citrate), 5x Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μ g/mL salmon sperm. Filters were washed to a stringency of 0.1% SSC at 65°C and exposed to Kodak XAR film. Autoradiograms were developed at different exposures and scanned by densitometry.

DNA Probes

The *c-myc* DNA representing either exon II (Pst-Pst, 414 base pair [bp]) or exon III (ClaI-EcoRI, 1401 bp) were generated from the plasmid pHSRI.¹⁶ The β -actin DNA (EcoRI-BamHI, 700bp) was obtained from the pHF β A-3'ut plasmid,¹⁷ the heat shock DNA (BamHI-EcoRI, 800 bp) from the pUR-HS70 plasmid,¹⁸ and the β -globin DNA (BamHI, 1.8 kb) from the p β 4.4 plasmid.¹⁹

[¹⁴C]-Uridine Incorporation

HL-60 cells (1×10^6 /mL) were exposed to either TNF α or cycloheximide (CHX) in 24 well plates in quadruplicates per experimental point for four hours. For combined treatment, CHX was added 30 minutes before TNF α . The cells were pulsed with 0.5 μ Ci of [¹⁴C]-uridine for two hours at 37°C, washed twice in phosphate-buffered saline (PBS), precipitated in 5% trichloroacetic acid (TCA) for ten minutes, washed twice in 5% TCA, and heated at 80°C for 60 minutes. A total of 200 μ L of each sample was counted by liquid scintillation.

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Nuclear Transcription

For run-on experiments nuclei were isolated by resuspending the cells in hypotonic buffer (10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L KCl, 3 mmol/L MgCl₂) and lysing the cells with Nonidet P-40. The nuclei were washed twice in hypotonic buffer, resuspended in nuclear storage buffer (40% glycerol, 50 mmol/L Tris-HCl [pH 8.3], 5 mmol/L MgCl₂, 0.1 mmol/L EDTA) and stored at -70°C. Transcription of nuclei and RNA isolation were performed as described.²⁰ The nuclei (1×10^8) were thawed and incubated for 10 minutes at 26°C in reaction buffer containing 200 μ Ci [³²P]-UTP (3,000 Ci/mmol), and the labeled RNA was hybridized for three days to Southern blots of restriction-enzyme digests of the various clones.^{16,17,19} The blots were washed to a final stringency of $0.1 \times$ SSC, 0.1% SDS and washed again at room temperature for 30 minutes in RNase (10 μ g/mL, in 2x SSC). The filters were washed again at room temperature for 15 minutes in 2x SSC and exposed to Kodak XAR films.

RESULTS

Regulation of *c-myc*-Messenger RNA Accumulation by TNF α in HL-60 Cells

Time-response studies. HL-60 cells were exposed for various lengths of time (15 minutes to four hours) to TNF α (100 U/mL; Fig 1A). Total RNA was isolated and analyzed by Northern blot technique. The strongest band of hybridization was detected at 2.5 kilobase (kb) when a DNA probe representing Exon II of the *c-myc* gene was used. This is consistent with previously reported data. A second, smaller transcript was seen at 1.6 kb, as observed by other investigators.^{21,22} A four-hour exposure to TNF α (100 U/mL) reduced *c-myc* messenger RNA (mRNA) accumulation in HL-60 cells by 95%, and a 50% reduction occurred within <one hour (Fig 1A, upper panel). No change in viability of the cells occurred, as determined by trypan blue exclusion. Hybridization of the same blot with a β -actin DNA probe demonstrated no change in mRNA levels at each of the time points (Fig 1A, lower panel); and no change in mRNA levels was observed when the same blot was rehybridized with a heat shock cDNA probe¹⁸ (data not shown). This shows that TNF α does not nonspecifically alter mRNA turnover in HL-60 cells.

Dose-response studies. Exposure of HL-60 cells for four hours to various concentrations of TNF α (0.1 to 100 U/mL) revealed a dose-dependent decrease in *c-myc*-mRNA levels (Fig 1B); a 50% reduction occurred in the range of 15 U/mL. These results were similar whether the same Northern blot was hybridized with a DNA probe representing either exon II (upper panel) or exon III (lower panel) of the *c-myc* gene. No change in viability of the cells occurred, as determined by trypan blue exclusion.

Effect of TNF α on *c-myc* Expression in Different Cell Types

Several different experiments were performed to examine the effect of TNF α on *c-myc* expression in cells with different cell growth responses to TNF α . First, a variant cell line of HL-60, which is resistant to the growth inhibiting action of TNF α , was established. The growth inhibitory effect of TNF α was determined by the clonogenic assay in

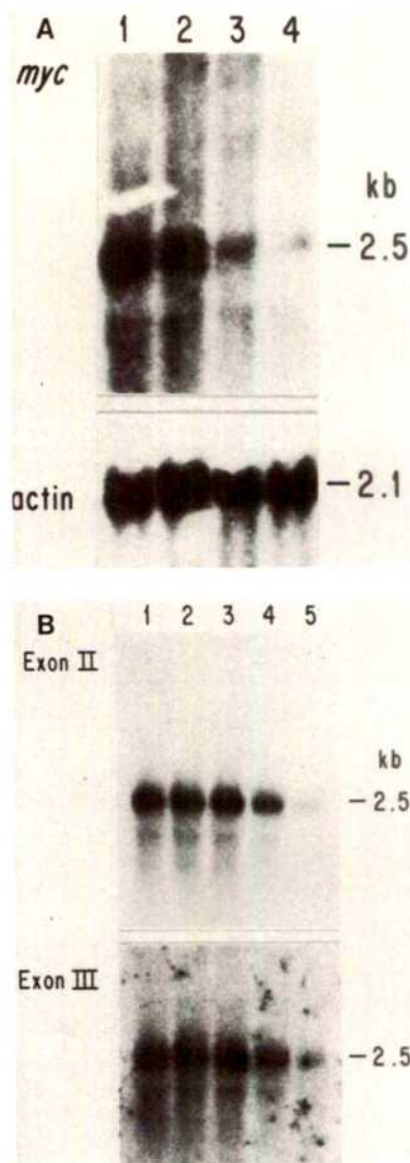


Fig 1. Regulation of *c-myc*-mRNA levels by TNF α in HL-60 cells. (A) Time-response studies. Upper panel: Northern blot of total RNA (20 μ g/lane) from HL-60 cells hybridized with a *c-myc* DNA probe (Exon II); HL-60 cells were exposed for various times to TNF α (100 U/mL); lane 1, control (no TNF α); lane 2, 15-minutes exposure to TNF α ; lane 3, one-hour exposure; lane 4, four-hour exposure. Lower panel: Autoradiogram of hybridization of the same blot with β -actin. (B) Dose-response studies. Upper panel: Hybridization with a *c-myc* DNA probe (Exon II) to total HL-60 mRNA (20 μ g/lane) isolated after treatment for four hours with various concentrations of TNF α . Lane 1, control HL-60 cells; lane 2, HL-60 cells exposed to 0.1 U/mL TNF α ; lane 3, cells exposed to 1 U/mL; lane 4, 10 U/mL; lane 5, 100 U/mL. Lower panel: Hybridization of the same blot with a DNA probe of exon III of the *c-myc* gene. RNA analysis was performed as described in Materials and Methods.

soft agar.¹¹ When both the HL-60 variant and control cells were plated in the presence of TNF α (500 U/mL) in soft agar, no decrease in the colony numbers could be observed with the HL-60 variant cells, whereas a profound reduction in colony numbers occurred in HL-60 control cells (0% and

>99% reduction of colony formation respectively, as compared to the untreated control samples). Exposure of HL-60 variant cells to TNF α (100 or 500 U/mL) for four hours did not reduce *c-myc*-mRNA levels (Fig 2A). The viability of the cells before and after the experiments was >95% as determined by trypan blue exclusion.

Second, recent studies showed that TNF α enhances proliferation of normal fibroblasts from several tissues such as diploid skin (Detroit 551) and lung (WI-38) fibroblasts.²³ Normal human lung fibroblasts were exposed for one and four days to TNF α (500 U/mL), which resulted in an increase of *c-myc*-mRNA accumulation (2.7-fold increase as compared to fibroblasts not cultured with TNF α ; Fig 2B).

Influence of New Protein Synthesis on the Regulation of *c-myc* Expression by TNF α

To analyze the role of protein synthesis in the regulation of *c-myc*-mRNA accumulation by TNF α , the HL-60 cells were exposed for various lengths of time to either CHX 20 μ g/mL, a protein synthesis inhibitor; TNF α (100 U/mL); or TNF α in combination with CHX (Fig 3A). A short exposure (45 min) to CHX alone increased *c-myc*-mRNA levels by 2.5-fold (data not shown), which is in agreement with previously reported data.^{4,24,25} Pretreatment with CHX for 30 minutes and addition of TNF α for two hours resulted in a very profound decrease in *c-myc*-mRNA accumulation (>95% reduction compared with the untreated HL-60 controls) (Fig 3A, lane 4). CHX (2.5 hours, lane 2) and TNF α (two hours, lane 3) alone reduced *c-myc*-mRNA levels <10% and 90% respectively, compared with untreated HL-60. No *c-myc* mRNA was detectable even after a seven-day exposure of the autoradiogram when HL-60 cells were treated for four hours with TNF α in combination with a pre-exposure to CHX for

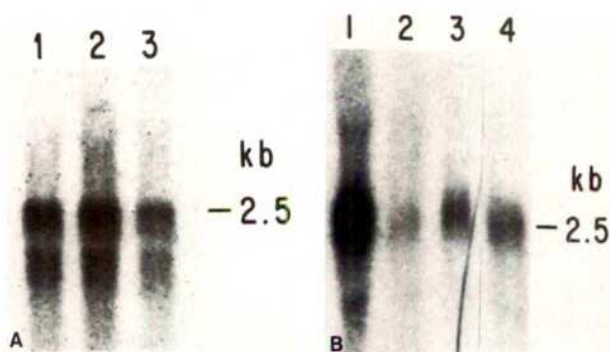


Fig 2. *c-myc*-mRNA accumulation in both a TNF α -resistant HL-60 variant cell line and normal lung fibroblasts after exposure to TNF α . (A) *c-myc*-mRNA levels in HL-60 variant cells, which are resistant to the cytotoxic effect of TNF α . Total RNA was isolated after a four-hour exposure of cells to TNF α at the concentrations of 100 U/mL (lane 2) and 500 U/mL (lane 3); lane 1 represents the total RNA from the untreated, TNF α -resistant HL-60 control cells. RNA was hybridized with [³²P]-labeled *c-myc* DNA (exon II) by Northern blotting. (B) Northern blot of normal lung fibroblasts exposed to TNF α . Cytoplasmic RNA was extracted after the cells were cultured with TNF α (500 U/mL) for one day (lane 3) and four days (lane 4). Lane 1, HL-60 RNA; lane 2, fibroblast RNA from the untreated control sample. Each lane in A and B contains 20 μ g RNA. Further analysis was performed as described in Materials and Methods.

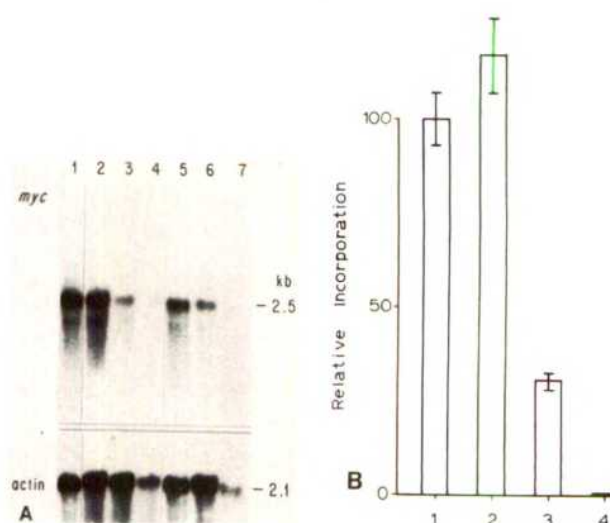


Fig 3. Influence of the inhibition of protein synthesis on the action of TNF α in HL-60 cells. (A) *c-myc*-mRNA levels in HL-60 cells after treatment for various durations with TNF α (100 U/mL) and the protein synthesis inhibitor cycloheximide (CHX, 20 μ g/mL). Analysis was performed by blotting total RNA (20 μ g/lane) from HL-60 cells and hybridizing with a *c-myc* DNA probe (Exon II) as described in Materials and Methods. Lane 1, untreated control sample (no TNF α , no CHX); lanes 2, 5, RNA from HL-60 cells treated with CHX for 2.5 hours and 4.5 hours, respectively; lanes 3, 6, RNA isolated from HL-60 cells treated with TNF α (100 U/mL) for two hours and four hours, respectively; lanes 4, 7, RNA of the cells after treatment with CHX for an initial 30 minutes plus TNF α for an additional two hours and four hours, respectively. (B) [¹⁴C]-uridine incorporation by HL-60 cells after treatment with TNF α (100 U/mL) and CHX (20 μ g/mL) as a measurement of RNA metabolism. Analysis was performed as described in Materials and Methods. [¹⁴C]-uridine incorporation of: Bar 1, HL-60 control cells (no TNF α , no CHX); bar 2, HL-60 cells cultured for four hours with TNF α alone; bar 3, HL-60 cells cultured for four hours with CHX alone; bar 4, HL-60 cells treated with TNF α (four hours) plus CHX (4.5 hours). Results are expressed as relative incorporation (mean $\pm$ SD) as compared to the control HL-60 not treated with CHX or TNF α . Incorporation was measured in cpm; absolute values for the control samples were in the range of 0.957 to 1.236×10^6 cpm.

30 minutes (lane 7). CHX (4.5 hours, lane 5) and TNF α alone (4 hours, lane 6) decreased *c-myc* mRNA levels by 60% and 90% respectively. Control experiments using a β -actin DNA probe showed no changes in mRNA levels in HL-60 cells cultured in the presence of either CHX or TNF α alone (Fig 3A). However, a marked decrease in β -actin mRNA levels was observed in cells treated with both CHX and TNF α for two and four hours (lanes 4, 7). At each experimental point >97% of the cells were viable as determined by trypan blue exclusion.

A greater than 99% decrease of total RNA synthesis in HL-60 cells cultured for four hours with TNF α (100 U/mL) and CHX (20 μ g/mL) occurred compared with control cells, as measured by [¹⁴C]-uridine incorporation into trichloroacetic acid precipitable counts (Fig 3B). Exposure to CHX alone for four hours resulted in a 70% decrease of [¹⁴C]-uridine incorporation, whereas a slight increase (17%) was seen in these cells treated with TNF α alone for four hours. These results suggest that (1) new protein synthesis is

required for continued production of *c-myc* mRNA (Fig 3A, lanes 2, 5); (2) TNF α reduces steady-state *c-myc*-mRNA levels independently of new protein synthesis (Fig 3A, lanes 4, 7); (3) TNF α has a marked effect on general turnover of RNA in HL-60 cells when new protein synthesis is suppressed by CHX (Fig 3A, lanes 4, 7; Fig 3B, bar 4); and (4) the general transcriptional activity of HL-60 cells is not reduced by TNF α at the concentration tested (Fig 3B, bar 2), but the same concentration of TNF α has a profound effect on *c-myc*-mRNA level (Fig 3A, lanes 3, 6).

Mechanism of Control of *c-myc* Expression by TNF α

Decreased levels of *c-myc* mRNA in HL-60 cells exposed to TNF α could result from either a reduced rate of transcription or a destabilization of the *c-myc* mRNA (post-transcriptional regulation). The transcriptional regulation of *c-myc* expression by TNF α was studied by an in vitro transcriptional run-on assay. In this assay in vitro transcripts are generated by elongation of previously initiated RNA chains during incubation of isolated nuclei in the presence of [³²P]-UTP.²⁶ This measurement of in vitro chain elongation reflects the numbers of polymerase molecules actively involved in transcription before cell lysis. The run-on transcripts were hybridized to DNA immobilized on nylon-membrane filters. As shown in Fig 4A (lane 1), exposure of HL-60 cells to TNF α (100 U/mL) for four hours resulted in a profound decrease in the transcriptional rate of the *c-myc* (85% reduction as compared to untreated HL-60 cells). In the same experiments β -actin DNA (lane 2) was used as control. No decrease in β -actin transcripts was observed, demonstrating that comparable conditions were used for both experimental points. No hybridization could be detected to β -globin DNA (lane 3), showing that nonspecific hybridization did not occur to a DNA, whose gene expression is not detectable in HL-60 cells. The size separation of the respective *c-myc*, β -actin, and β -globin inserts from the vector DNA (4.4 kb) by gel electrophoresis further excludes a nonspecific hybridization to plasmid DNA. Viable cell counts did not change in the HL-60 cells exposed to TNF α (100 U/mL $\times$ 4 hours), as compared to control HL-60 cells.

Post-transcriptional regulation was studied by examining changes in *c-myc* mRNA at each time point of cell harvest. If TNF α mediated a post-transcriptional regulation of *c-myc*, the levels of *c-myc* mRNA would be lower in the TNF α plus actinomycin D-treated cells as compared with cells treated with actinomycin D alone. Actinomycin D blocked >98% of all transcription in HL-60 cells as determined by [¹⁴C]-uridine incorporation. As shown in Fig 4B, no change in *c-myc*-mRNA levels was detectable in HL-60 cells treated with TNF α plus actinomycin D as compared to cells treated with actinomycin D alone. Therefore TNF α probably has no effect on the stability of *c-myc* mRNA in HL-60 cells.

DISCUSSION

Both transcriptional and post-transcriptional mechanisms regulate *c-myc*-mRNA levels in human cells. A post-transcriptional regulation accounted for the decreased

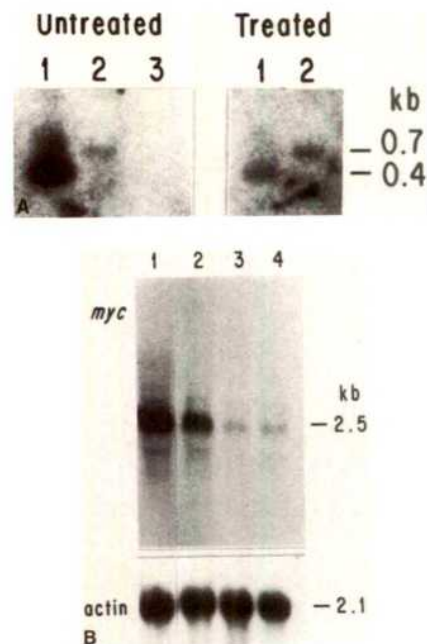


Fig 4. (A) Transcriptional analysis of *c-myc* in isolated nuclei of HL-60 cells untreated or treated with TNF α (100 U/mL) for four hours. Analysis was performed as described in Materials and Methods. Autoradiograms show hybridization of [³²P]-labeled transcripts to the *c-myc* DNA (Exon II; lane 1). β -actin (lane 2) and β -globin (lane 3) were used as positive and negative controls respectively. The DNA inserts were size separated from their plasmid vectors by a 1.2% agarose gel and transferred to a nylon-membrane filter. (B) *c-myc*-mRNA accumulation in HL-60 cells after treatment with TNF α (100 U/mL) and actinomycin D (5 μ g/mL) for 75 minutes. Analysis was performed by RNA blotting as described in Materials and Methods. Total RNA (20 μ g/lane) was hybridized with a probe of *c-myc* DNA (Exon II). RNA from: lane 1, untreated HL-60 cells; lane 2, HL-60 cultured with TNF α ; lane 3, HL-60 cultured with actinomycin D; lane 4, HL-60 cultured with TNF α plus actinomycin D. The lower panel shows an autoradiogram of control hybridization of the same blot with β -actin.

c-myc-mRNA accumulation both in lymphoblasts treated with gamma-interferon^{27,28} and in F9 embryonal carcinoma cells induced to differentiate with chemicals.^{29,30} By contrast, a block to elongation of *c-myc* mRNA was shown to be responsible for the reduced transcription in HL-60 cells induced to differentiate with all-*trans*-retinoic acid.²¹ We demonstrated in the present paper that TNF α rapidly and sensitively inhibited the expression of the *c-myc* gene at the level of transcription in HL-60 cells (Figs 1A, 1B, 4A and 4B). By contrast, rate of transcription of β -actin remained unchanged, and the overall viability of HL-60 was not affected by TNF α . We also showed by using [¹⁴C]-uridine incorporation as a measurement of total RNA metabolism that TNF α did not decrease the overall transcriptional activity of HL-60 cells in the conditions that we employed (100 U/mL $\times$ four hours; Fig 3B); in fact, TNF α slightly stimulated [¹⁴C]-uridine incorporation. These results indicate that TNF α does not decrease nonspecifically the general transcriptional rate of HL-60 cells.

The TNF α produced a half-maximal inhibition of *c-myc* expression in the range of 15 U/mL after a four-hour

exposure to TNF α (Fig 1B). We recently showed that TNF α sensitively inhibited clonal growth of HL-60 cells in soft agar; and a 50% inhibition (ED₅₀) of clonal growth occurred in the range of 6 U/mL.¹ In this assay the cells were exposed for ten days to TNF α . Pulse-exposure experiments demonstrated that a three-hour exposure to 20 U/mL TNF α resulted in a 50% inhibition of HL-60 clonal growth. This nearly parallels the decrease in *c-myc*-mRNA levels in HL-60 cells (Figs 1A, 1B).

Our study using the TNF α -resistant HL-60 variant cell line demonstrated that TNF α does not decrease *c-myc*-mRNA levels in cells that are resistant to the growth-inhibiting action of TNF α (Fig 2A); and with normal lung fibroblasts, whose growth is enhanced by TNF α , the monokine actually increased the expression of *c-myc* mRNA (Fig 2B). Thus our results suggest an inverse association between growth inhibition mediated by TNF α and levels of *c-myc* mRNA in these cells.

The recent literature provides some controversial data about the effect of TNF α on HL-60 cells. Using early passages (15 to 20) of HL-60 cells, we found that TNF α very sensitively inhibited clonal growth of HL-60 cells in soft agar,¹ and in the present paper we demonstrate that TNF α reduces *c-myc* transcription in these cells in a parallel manner. By contrast, Peetre et al³¹ recently reported that wild-type HL-60 cells were highly resistant to the growth inhibition by TNF α in soft agar. However, a subclone of HL-60 cells was very sensitive to the inhibitory action of TNF α in the same assay. These discrepancies in the effects of TNF α on HL-60 cells might be more apparent than real and may result from the utilization of different sublines and passages of HL-60 cells or different TNF α preparations.

Furthermore we found that the action of TNF α on *c-myc* is not dependent on new protein synthesis (Figs 3A, 3B). Blockade of protein synthesis in HL-60 by CHX and treatment of the cells with TNF α resulted in a further decrease of *c-myc*-mRNA levels, compared with the levels in cells treated with either CHX or TNF α alone. In addition, TNF α

plus CHX markedly reduced steady-state mRNA levels of β -actin, while both TNF α and CHX alone had no effect on β -actin mRNA levels (Figs 3A). These results suggest that TNF α and CHX interact in a synergistic fashion, and in the absence of new protein synthesis, TNF α profoundly affects overall steady-state mRNA in HL-60 cells.

The mechanism by which TNF α exerts its growth-inhibiting activity is unknown. TNF α interacts with the cell through a single class of specific TNF α receptors.³²⁻³⁷ The means by which receptor-bound TNF is toxic to the cell is unclear. Experiments with inhibitors of TNF α suggest that internalization of the TNF α -receptor complex may be critical for its effect.³⁸ One possibility might be that, similar to the glucocorticoids, the TNF α -receptor complex specifically binds to certain DNA sequences in the region of *c-myc* and inhibits the transcription of the gene. Another possibility is that the TNF α -TNF α receptor complex activates a second messenger that directly or indirectly affects transcription of *c-myc*. Recent studies showed that the *c-myc* protein may be part of the DNA polymerase complex and is necessary for DNA synthesis in HL-60 cells.³⁹ TNF α , by inhibiting the transcription of *c-myc*, causes a decrease of *c-myc* protein levels, which in turn might contribute to the reduced proliferation and survival of HL-60 cells and neoplastic cells in general. Yet further studies are necessary to determine whether down-regulation of *c-myc* is a major cause of the growth inhibition of TNF α or is a secondary phenomenon.

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NOTE ADDED IN PROOF

After submission of this paper, Krönke et al also reported a transcriptional regulation of *c-myc* by HL-60 cells.⁴⁰

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Recombinant Human GM-CSF Induces Leukocytosis and Activates Peripheral Blood Polymorphonuclear Neutrophils in Nonhuman Primates

By P. Mayer, C. Lam, H. Obenaus, E. Liehl, and J. Besemer

The *in vivo* efficacy of glycosylated and nonglycosylated recombinant human granulocyte macrophage colony-stimulating factor (rh GM-CSF) expressed in Chinese hamster ovary cells and *Escherichia coli* respectively was studied in rhesus monkeys following a daily subcutaneous (SC; three times) or intravenous (IV; over six hours) dose for seven consecutive days. The monkeys responded to the rh GM-CSF with a prompt (within 24 hours) rise in circulating white blood cells (WBCs). Thereafter the total cell counts increased steadily in a dose-dependent manner with repeated dosing to numbers six times over the pretreatment levels. Overall, granulocyte counts increased fivefold, lymphocytes twofold to fourfold, and monocytes threefold to fourfold. Platelets and erythrocytes were unaffected.

THE *IN VITRO* proliferation, differentiation, and functional activation of hematopoietic progenitor cells is dependent on the presence of a family of glycoproteins known as colony stimulating factors (CSFs).^{1,2} These factors are classified according to the types of hematopoietic progenitor cells with which they interact. GM-CSF stimulates the growth and differentiation of granulocyte and macrophage precursors, while G-CSF stimulates the terminal differentiation of granulocyte precursors, and M-CSF is thought to be responsible for the growth and differentiation of macrophage progenitors. The molecular biology and function of CSFs have been reviewed recently.¹

The molecular cloning of the complementary DNA (cDNA) for human GM-CSF has made it possible to determine the biological activities of the purified recombinant material in *in vitro* and *in vivo* test systems.^{3,5} The recombinant human GM-CSF (rh GM-CSF) stimulates the formation of macrophage, granulocyte, and eosinophil colonies in human bone marrow cell cultures.⁶ It also increases erythroid and multipotential colony formation in enriched human bone marrow and peripheral blood progenitor cell cultures containing erythropoietin.⁷⁻¹⁰ In addition, rh GM-CSF was able to prime mature human granulocytes for increased phagocytosis and eosinophils for elevated autofluorescence.^{3,9,11} Rh GM-CSF had no proliferative effects on murine hematopoietic cells³ but regulates the formation of granulocyte, macrophage, erythroid, and mixed colonies in nonhuman-primate bone marrow cultures.¹² This suggests that the *in vivo* activity of rh GM-CSF can be evaluated in nonhuman primates. Recently it was reported that continuous infusion

Within 1 week after the end of treatment the leukocytosis had disappeared. Of the two routes of treatment, SC (three times daily)-administered rh GM-CSF was more effective than the same dose given by a six-hour IV infusion. In addition to inducing leukocytosis, parenterally administered rh GM-CSF primed mature circulating granulocytes for enhanced oxidative metabolism and killing of an *E coli* strain. These results show that exogenously administered glycosylated or nonglycosylated rh GM-CSF is both an effective stimulator of leukocytosis and a potent activator of the phagocytic function of mature granulocytes in monkeys.

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of a highly glycosylated form of the rh GM-CSF in monkeys elicited a dramatic leukocytosis.¹² The rh GM-CSF used in this primate study was expressed in genetically engineered Chinese hamster ovary (CHO) cells and had a similar degree of glycosylation as the natural product.

The purposes of the present study were: (1) to confirm the reported *in vivo* efficacy of the glycosylated rh GM-CSF by a route of administration other than continuous infusion; (2) to determine if nonglycosylated material is also active *in vivo*; and (3) to establish whether or not peripheral neutrophils become activated following *in vivo* administration of rh GM-CSF.

MATERIAL AND METHODS

Animals

Nine male and five female adult rhesus monkeys (*Macaca mulatta*) approximately 4- to 8-years old and weighing between 5 and 13 kg were kept individually in monkey cages in an air-conditioned room at 25°C. The animals were fed with certified primate pellets. The animals selected for the intravenous (IV) infusion studies were trained to sit in restraining chairs prior to the experiment.

Sources of rh GM-CSF

The glycosylated rh GM-CSF was purified from transfected CHO cells, as described elsewhere,^{3,4} and was provided by Genetics Institute, Cambridge, MA. The purity of the material, determined by Coomassie blue staining after digestion with N-glycanase, was 96.9%. It had a specific *in vitro* biological activity of 2×10^6 U/mg protein when assayed by thymidine incorporation by human peripheral blood myeloblast (CML) cells,¹³ and 1 to 2×10^5 U/mg protein when measured with a radioreceptor binding assay (manuscript in preparation). The nonglycosylated rh GM-CSF was extracted from *Escherichia coli* cells expressing the GM-CSF cDNA from a plasmid vector. The protein was accumulated intracellularly. *E coli*-derived rh GM-CSF was purified to homogeneity by a series of chromatographic steps, including high-performance liquid chromatography (HPLC), and had a specific activity of 2 to 4×10^7 U/mg protein in the CML bioassay and 5 to 10×10^5 U/mg protein in the radioreceptor assay (manuscript in preparation). The purity of this material was 99%, determined by HPLC analysis.

Endotoxic activity was determined in the rh GM-CSF preparation using a chromogenic *Limulus* amoebocyte lysate reagent kit (Whittaker, M.A., Bioproducts, Inc, Walkersville, MD).

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Administration of rh GM-CSF to Monkeys

The IV infusion solutions were prepared by injecting the appropriate amount of the sterile filtered rh GM-CSF into infusion bags containing nonpyrogenic saline solution (Natrium chloratum physioli; Flex Flac, Leavosan, Austria). The infusion bags were prepared individually for each IV-treated monkey and contained 25 mL saline/kg body weight. To avoid nonspecific binding of rh GM-CSF to the plastic surfaces of the infusion devices, monkey serum at a concentration of 0.5% was added. The solutions were administered for seven consecutive days over a period of six hours and at a flow rate of approximately 1 mL/min.

The rh GM-CSF solution for the subcutaneous (SC) treatments was prepared by diluting the daily dose with nonpyrogenic saline containing 0.5% monkey serum to 15 mL. Five milliliters of the rh GM-CSF solution was administered SC at different sites in the back at 8 AM, 1 AM, and 6 PM each. The administrations were performed over a period of seven days.

The control monkeys received IV infusions or SC injections of nonpyrogenic saline with 0.5% monkey serum containing the same amount of endotoxin (LPS World Health Organization standard from *E. coli* J5) detected in the high dose of the rh GM-CSF preparation.

Hematologic Examinations

Blood was collected for hematologic examinations in EDTA-coated tubes during the pretreatment week on days -8 and -6. During the treatment period blood samples were collected every day prior to the infusions or SC injections (day 0 to day +6). In the post-treatment period blood was sampled three times a week for 3 weeks.

The hematologic examinations included the total counts of red blood cells (RBCs) and white blood cells (WBCs), determination of hemoglobin and hematocrit (Microcell counter CC 108, TOA; Tokyo, Japan) of platelets (Platelet counter PL 100, TOA; Tokyo, Japan) and reticulocytes (Brilliant cresyl blue staining), and differential blood count (Wright's stain).

Assay of Peripheral Blood Polymorphonuclear Neutrophil (PMN) Functions

PMNs were fractionated from heparinized venous blood by Ficoll-Hypaque gradient centrifugation and dextran sedimentation (99% pure and viable as evaluated by Giemsa stain and Trypan exclusion test). Superoxide anion generation was measured in response to the bacterial chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (f-MLP).¹¹ Briefly 5×10^6 polymorphonuclear cells (PMNs) suspended in 0.2 mL Hanks' balanced salt solution (HBSS) were added to each of two tubes containing 1.2 mg cytochrome C per mL. Superoxide dismutase was added to one tube, and f-MLP was added to both tubes at a final concentration of 10^{-8} mol/L. The cell suspensions were incubated at 37°C for 15 minutes, and superoxide dismutase inhibitable cytochrome C reduction was measured in a spectrophotometer at 550 nm (Ultrospec 4050 LKB, Sweden). The data were expressed as percent increase in the amount of O_2^- released by PMNs from monkeys during and after treatment with rh GM-CSF compared to values obtained from cells collected before treatment.

The bactericidal activity of PMNs was determined in whole blood by adding *E. coli* Δ 1060 to heparinized blood to give a bacteria-neutrophil ratio of 1. The mixture was shaken in a water bath at 37°C for two hours. After lysing the blood cells with 0.1% Triton X-100, the surviving organisms were estimated by plating various dilutions of the homogenates on Trypticase soy agar plates, incubating at 37°C for 24 hours, and counting the colony-forming units

(CFUs). The survival of the organisms in pooled fresh monkey serum was also evaluated in parallel.

Morphologic Examination of Bone Marrow Aspirates

Bone marrow samples were obtained by aspiration from the posterior ilial crest under general anesthesia (Ketalar, 20 mg/kg intramuscularly [IM]) prior to treatment and during the drug treatment period. Bone marrow smears were prepared and stained according to Pappenheim.^{13a} Differential cell count was performed by examining 200 white cells. In addition, the red cells were also recorded. The number of white and red cells was calculated in percent.

RESULTS

IV Infused Glycosylated rh GM-CSF-Induced Leukocytosis

Daily IV infusion of 253, 84, and 28 μ g glycoprotein rh GM-CSF per kilogram body weight in monkeys over seven days induced a prompt rise in total WBC counts in circula-

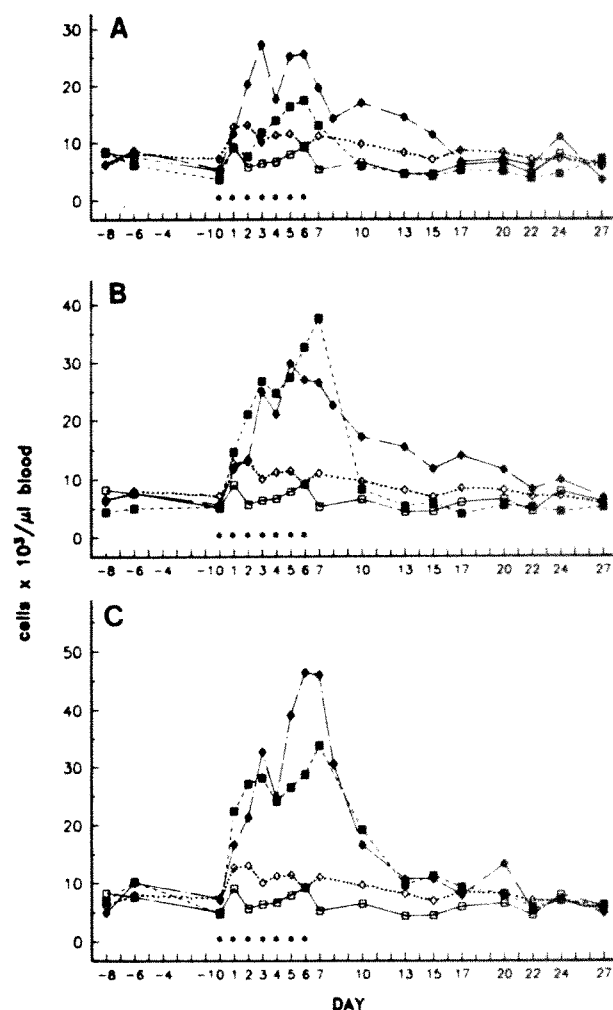


Fig 1. Changes in peripheral blood levels of WBCs in rhesus monkeys infused with glycosylated recombinant human GM-CSF (rh GM-CSF) for seven days (*). Filled symbols: Monkeys infused daily with 28 μ g/kg (A), 84 μ g/kg (B), or 253 μ g glycoprotein/kg (C). Open symbols: Control animals infused with saline containing 7×10^{-9} mg endotoxin/kg.

Table 1. Maximum Levels of WBC, Neutrophils, Eosinophils, Monocytes, and Lymphocytes in Peripheral Blood of Rhesus Monkeys in Response to rh GM-CSF Treatments

Source of GM-CSF	Doses μg Protein/ Glycoprotein/ kg body weight	Route of Administration	WBC $\times 10^3/\mu\text{L}$ Blood		Segmented Neutrophils $\times 10^3/\mu\text{L}$		Eosinophils $\times 10^3/\mu\text{L}$		Monocytes $\times 10^3/\mu\text{L}$		Lymphocytes $\times 10^3/\mu\text{L}$	
			Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2	Animal 1/Animal 2
Control	—		9.1	11.4	7.3	7.4	0.7	0.7	0.1	0.2	3.4	3.3
CHO-derived material (glycosylated)	16/28	IV infusions	17.3	27.1	12.0	20.3	1.6	1.8	0.5	0.8	4.7	4.8
	50/84	6 hours	37.6	29.8	23.3	22.0	4.5	1.6	1.0	0.5	5.9	5.6
	150/253	each for 7 days	33.7	46.3	23.0	31.4	3.4	7.8	1.0	0.9	5.4	7.4
	1.7/2.9		19.0		7.8		3.5		0.3		6.3	
	5/8.5		32.5		14.3		4.2		0.4		14.0	
CHO-derived material (nonglycosylated)	15/25.3		33.9		16.7		2.3		1.0		11.8	
	50/84	SC injections for 7 days; daily dose divided into 3 applications	62.4	62.5	45.5	48.1	3.1	2.8	3.7	2.1	9.5	9.7
Control	—		12.9		5.5		0.4		0.2		7.3	
<i>E. coli</i> -derived material (nonglycosylated)	1.7	SC injections	26.8		8.7		1.8		0.2		12.9	
	5	for 7 days; daily dose	31.3		14.0		2.4		0.5		14.3	
	15	divided into 3 applications	52.3	46.5	31.2	29.0	8.4	8.2	2.0	1.6	18.2	10.1
	50		53.7	53.1	30.0	40.4	16.6	7.4	2.3	4.0	14.6	12.0
	150		43.3		23.3		6.4		1.7		20.8	

NOTE. Monkeys were treated daily either SC or by IV infusion with various doses of glycosylated or nonglycosylated GM-CSF over a period of seven consecutive days. Blood was collected daily, and WBC and differential counts were performed. Maximum WBC levels were reached on the last day of the treatment period or the day after.

tion (Fig 1 A to C). The leukocytosis appeared within 24 hours of the first infusion and increased steadily, with repeated dosing reaching maximum levels on the last day or one day after the termination of the treatment. The rise in WBC counts was dependent on the dose of the infused rh GM-CSF. In Table 1 the maximum levels of WBC counts of the monkeys infused with the various amounts of rh GM-CSF are listed. Even with the low dose of 28 μg glycoprotein/kg/d, a twofold increase in the maximum WBC count over the values of control animals could be observed. With the higher doses of 84 μg and 253 μg glycoprotein/kg/d, a threefold to fourfold increase in WBC compared to control animals was achieved. The infusion of 84 μg glycoprotein/kg/d seems to be sufficient to reach plateau levels of the WBC increase. WBC counts began to decline on the first or second day after termination of treatments and had returned to control levels almost within a week. The period for recovery to normal WBC levels was not dose dependent. The control monkeys infused with saline containing the small amount of endotoxin determined in the high dose did not show a response in their WBC counts.

Effect of Glycosylated and Nonglycosylated rh GM-CSF on Leukocyte Counts After Subcutaneous Injections

Subcutaneously injected rh GM-CSF was more effective in eliciting leukocytosis than the same dose infused IV. Representative results shown in Table 1 indicate that a daily SC dose of 84 μg glycoprotein/kg produced WBC counts two times higher than that elicited by an IV infusion with the same dose. The rise in WBC counts in response to SC treatments appeared within 24 hours of the start of the

treatments and amounted to a twofold to threefold increase in circulating WBC counts over the preinfusion counts. Thereafter the WBC counts increased steadily, with repeated dosing up to the last day of treatment. As observed with IV infusions, the elevated WBC counts fall to preinfusion levels within four to six days after termination of the treatments (Fig 2A).

Thus the WBC counts in monkeys that received SC either the glycosylated or the nonglycosylated rh GM-CSF peaked at 62×10^3 and 53×10^3 per μL blood respectively (Figs 2A and 2B). Since 84 μg of the glycosylated material had a protein content of 50 μg , it can be concluded that the protein moiety is the biologically active part. An increase in the daily dose of the nonglycosylated material to 150 μg protein/kg or a decrease to a daily dose of 15 $\mu\text{g}/\text{kg}$ did not change the peak WBC levels significantly. Yet decreasing the daily dose to 5 μg protein/kg resulted in maximum WBC levels of 31×10^3 for the nonglycosylated and 32×10^3 for the glycosylated rh GM-CSF. A dose of 1.7 μg protein/kg elicited maximum WBC counts of 26×10^3 for the nonglycosylated form and 19×10^3 for the glycosylated material (Table 1). No clinical signs of toxicity were observed either in the IV-treated or in the SC-administered monkeys.

Again the small amounts of endotoxin detected in the high dose of rh GM-CSF and injected SC did not alter the WBC counts in control monkeys.

Effect of rh GM-CSF Treatment on Differential WBC Counts

Differential cell counts revealed that the increase in WBC counts was mainly due to the rise in segmented neutrophil

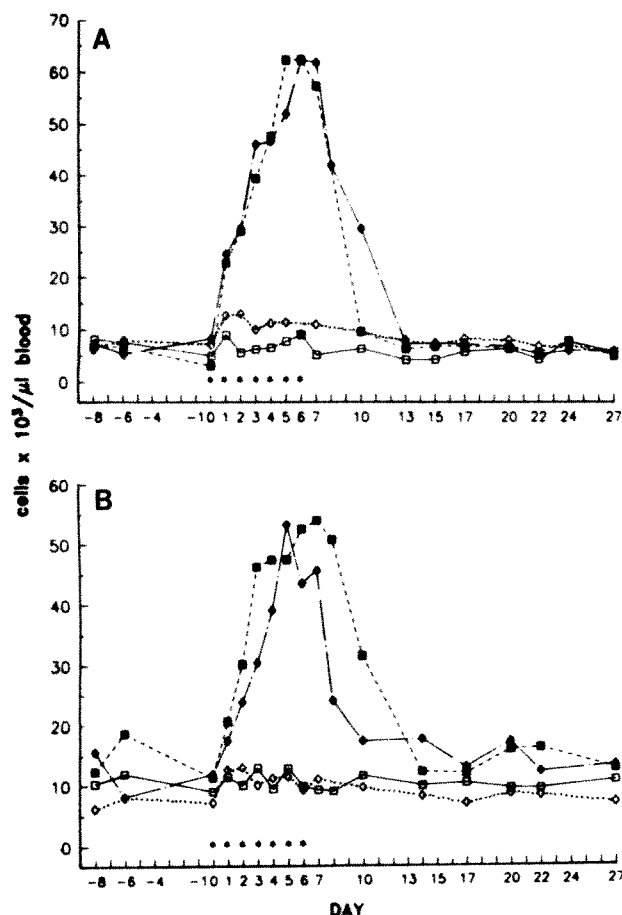


Fig 2. Changes in peripheral WBC levels in rhesus monkeys injected SC with glycosylated and nonglycosylated recombinant human GM-CSF (rh GM-CSF) for seven days (*). Filled symbols: Monkeys treated daily with 84 μ g/kg glycoprotein (A) or 50 μ g/kg protein (B). The daily dose was divided into three applications. Open symbols: Control animals injected with saline containing 7×10^{-9} mg endotoxin/kg.

numbers. The increase of segmented neutrophils paralleled the increase of the total WBC counts. The neutrophilia reached maximum levels on the last day of treatment with levels returning to normal values by days 2 to 5 after the termination of the treatments (Fig 3). Representative results in Table 1 show that the IV infusion of the low dose produced a more than twofold increase in neutrophil numbers compared to counts of the control monkeys. A threefold increase was observed in animals treated with the middle dose. The high-dose treatment did not increase the neutrophil counts further. The maximum neutrophil levels after SC treatments with the middle dose were twice as high as after IV infusions of the same dose (22.0 and 23.3 v 45.5 and 48.1 $\times 10^3$ neutrophils/ μ L blood). Therefore the three-times-daily SC administration of rh GM-CSF was more effective in increasing the neutrophil count than daily six-hour IV infusions.

Also a dose-dependent increase in the number of band cells was found. The increase and decline of band cell numbers showed a similar pattern as that of the segmented neutrophils. The most prominent increase in band cell levels was found in the SC-treated monkeys with a sevenfold to tenfold increase over control values (Fig 3).

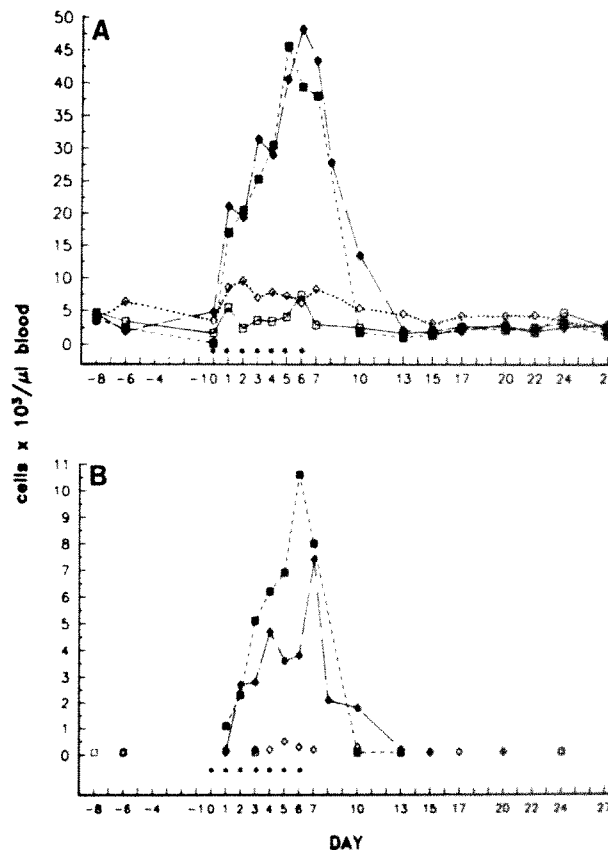


Fig 3. Changes in peripheral neutrophil counts in rhesus monkeys injected SC with glycosylated recombinant human GM-CSF (rh GM-CSF) for seven days (*). Filled symbols: Monkeys treated with a daily dose of 84 μ g glycoprotein/kg. The daily dose was divided into three applications. Open symbols: Control animals injected with saline containing 7×10^{-9} mg endotoxin/kg. (A), Segmented neutrophils; (B), band cells.

Effect of rh GM-CSF Treatment on Monocyte Count

Blood monocyte levels were also elevated in response to the rh GM-CSF treatment. In the IV-treated monkeys this increase seems to be dose dependent. Maximum monocyte levels were exhibited in monkeys treated with the middle and high dose. The highest monocyte levels developed in monkeys treated by the SC route (Fig 4).

Effect of rh GM-CSF Treatment on Eosinophil Count

In all the monkeys an eosinophilia developed in response to rh GM-CSF. The rise in eosinophil counts was delayed when compared to the prompt onset of neutrophilia in response to rh GM-CSF treatment. Nevertheless maximum eosinophil numbers coincided with the last day of the treatment period (Fig 4). When the maximum eosinophil levels were compared, the variation between animals treated with the same dose was more than twofold. Nevertheless it seems that in the IV-treated monkeys the maximum increase in eosinophil numbers was also dose dependent (Table 1).

Effect of rh GM-CSF Treatment on Lymphocyte Count

An increase in the lymphocyte count (twofold to threefold) was seen in the high-dose IV-treated monkeys but not in the

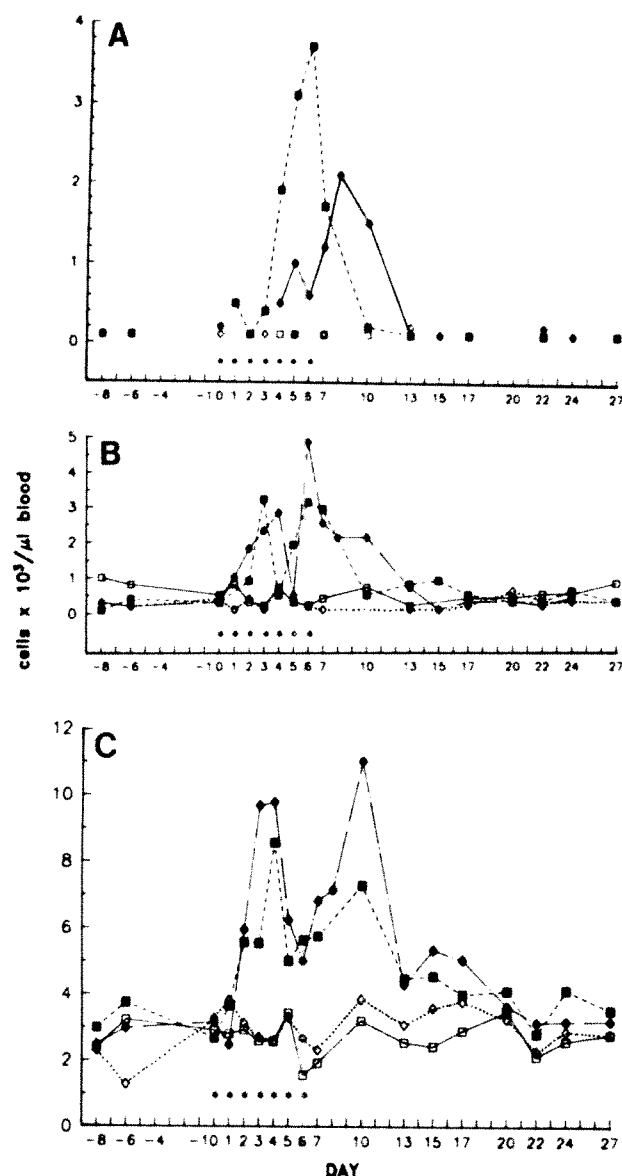


Fig 4. Changes in peripheral blood levels of (A) monocytes, (B) eosinophils, and (C) lymphocytes in rhesus monkeys injected SC with glycosylated recombinant human GM-CSF. Filled symbols: Monkeys treated with a daily dose of 84 $\mu\text{g/kg}$ glycosylated rh GM-CSF. The daily dose was divided into three applications. Open symbols: Control animals injected with saline containing 7×10^{-9} mg endotoxin/kg.

middle- and low-dose infused animals (Table 1). A lymphocytosis of the same magnitude was exhibited in the monkeys treated SC with the middle dose (Fig 4). Monkeys treated SC with increasing doses of the nonglycosylated material seem to respond with a dose-dependent lymphocytosis. The maximum lymphocyte counts were determined in the monkey treated with the high dose of 150 $\mu\text{g/kg/d}$ SC (Table 1).

The control animals that received IV infusions or SC injections containing an equal amount of endotoxin as the high dose monkeys did not show any of the changes in the lymphocyte count mentioned above.

Effect of rh GM-CSF Treatment on RBCs and Platelets

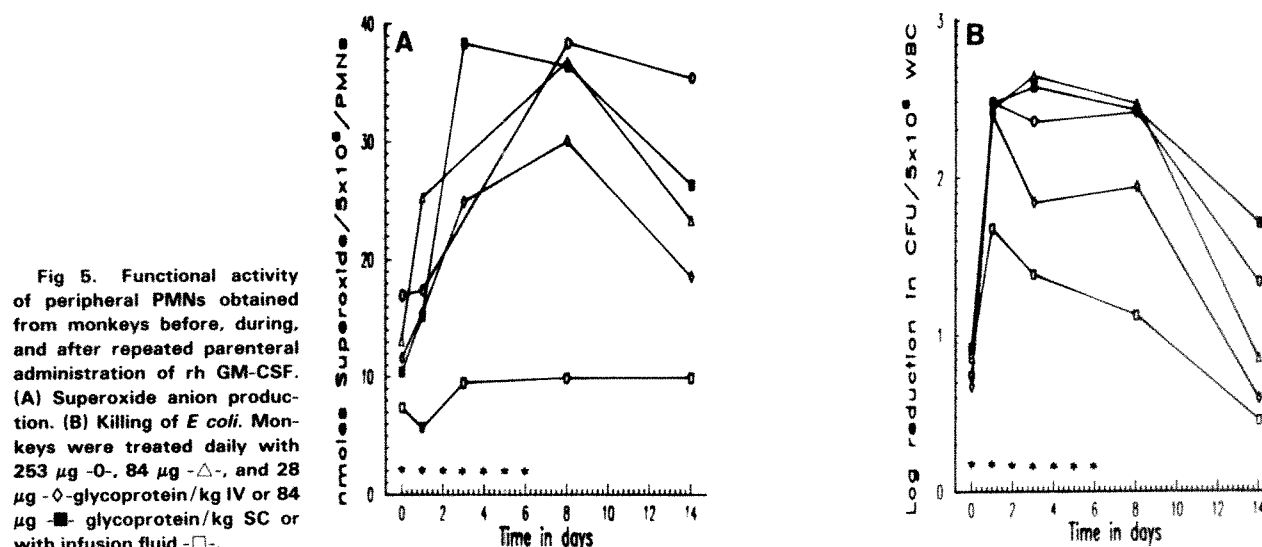
In all monkeys a slight decrease in RBC count, in the hemoglobin values, and in hematocrit were observed. This decrease coincided with the beginning of the treatment period, showed its nadir (10% to 20% reduction) in the first week of the post-treatment period, and had a tendency to recover at the end of the third week of the post-treatment observation period. With this decrease in these red blood parameters, a concomitant increase in reticulocyte numbers (twofold to threefold) was observed. These changes were found in control animals and can be interpreted as a slight anemia due to the repeated sampling of larger volumes of blood throughout the study. No changes were found in the platelet counts during and also after the treatment with rh GM-CSF in monkeys.

Morphological Examination of Bone Marrow of Monkeys Treated With rh GM-CSF

Bone marrow was aspirated before treatment, on the third day of the seven-day treatment period and again one day after termination of treatments. The morphological examination of the smears revealed that there was no difference in the percentage of neutrophils, eosinophils, lymphocytes, and monocytes between treated and control animals. Even in the SC-treated monkeys that showed a threefold increase in peripheral neutrophil numbers on the third day of treatment and a fivefold increase one day after termination of the applications, no changes in the percentage of neutrophils were found during and after rh GM-CSF treatment compared to bone marrow smears taken before treatment. Also no changes were found in the granulocyte precursors and also in cells of the erythroid lineage.

Effect of rh GM-CSF Treatment on PMN Function

Generation of superoxide anion. Since rh GM-CSF readily primes mature neutrophils *in vitro* for enhanced metabolism on exposure to a soluble stimulus, it is reasonable to expect that the first detectable parameter of *in vivo* efficacy would be the appearance of a population of neutrophils in the circulation with increased oxidative metabolism. To support this view, blood samples were collected immediately before and after rh GM-CSF infusion on the first and third days and two days after the seven-day infusion period and again on day 14 of the post-treatment period. The generation of superoxide anion by purified neutrophils was induced with 10^{-8} mol/L f-MLP and measured as superoxide dismutase inhibitable reduction of cytochrome C. As shown in Fig 5A, the rh GM-CSF treatment readily primed neutrophils *in vivo* for enhanced superoxide anion (O_2^-) release, with maximum effect occurring after the third infusion of the seven-day treatment period. The high rh GM-CSF dose did not produce a higher O_2^- release from the neutrophils than the middle dose. The GM-CSF-mediated enhancement of O_2^- release was maintained throughout the infusion period. The neutrophils obtained from control monkey were not primed for enhanced O_2^- release. It was also of interest to note that rh GM-CSF administered SC significantly improved the oxidative metabolism of the neutrophils.



Killing of *E. coli*

To evaluate whether the enhanced generation of O_2^- by the neutrophils observed also lead to increased killing of *E. coli*, the survival of the bacteria in blood from rh GM-CSF treated and untreated monkeys was studied. The results (Fig 5B) show that the killing of the *E. coli* increased by about 40 times in blood samples taken immediately after the first infusion. The enhanced antibacterial activity persisted throughout the infusion period. When rh GM-CSF was administered SC, the neutrophils in the peripheral blood were stimulated for enhanced antibacterial capacity to the same degree as during the IV infusions. The antibacterial activity of the neutrophils from the control monkeys also increased slightly, possibly because of the low amounts of endotoxin added to the infusion fluid.

DISCUSSION

The results of the present study indicate that purified rh GM-CSF is both an effective stimulator of leukocytosis and an activator of the phagocytic function of mature neutrophils in vivo. The repeated administration of rh GM-CSF to rhesus monkeys over a seven-day period produced a steady rise in peripheral WBCs with peak response occurring on the last day of the dosing. The rise in peripheral WBC was mainly a result of increased levels of circulating neutrophils, which contributed to about 70% to 80% of the leukocytosis. In addition, an increase in the numbers of eosinophils and monocytes that contributed 12% to 15% and 2% to 7% respectively for the increased WBC counts were observed.

The prompt rise in peripheral blood neutrophil levels in response to the infused rh GM-CSF suggests that besides its established role as a stimulator of hematopoiesis, GM-CSF acts also as a releasing factor for granulocytes from the marrow reserve pool. Stimulation of hematopoiesis by rh GM-CSF either at the stem cell or at the myeloblast level would not contribute to the increased numbers of circulating neutrophils until at least five days after the start of the treatment.¹⁴⁻¹⁶ The release of marrow granulocytes into the circulation would obviously be limited by the granulocytes

available in the marrow. The marrow granulocyte reserve pool was estimated to be three to seven times the size of the total blood granulocyte pool in humans. Estimates made to the contribution of the marrow reserve pool to increased circulating granulocyte counts suggest that when the circulating granulocytes double, the marrow reserve pool would last between 37 and 72 hours depending on its size. With a circulating granulocyte number four times normal, the marrow reserve would exhaust in 13 to 24 hours.¹⁷ If rh GM-CSF treatments would just increase the marrow-release rate of granulocytes in monkeys without changing the production, one would predict a decrease in the marrow granulocyte reserve and an exhaustion of the system. This exhaustion should also be followed by a decline in peripheral blood granulocyte counts beginning two to four days after starting treatments. But in contrast in the present study, granulocyte levels increased steadily up to the last day of the seven-day treatment period and remained elevated by treating a monkey as long as 21 days.¹² The important question of whether or not the rh GM-CSF acts only as a releasing factor or as a stimulator of hematopoiesis was also addressed by comparing bone marrow aspirates before and after rh GM-CSF administration. An exhaustion of the granulocyte reserve pool by stimulating the release of these cells into the circulation would have depleted granulocytes from the bone marrow. Yet bone marrow aspirates taken either during or after the treatment with rh GM-CSF showed no granulocyte depletion of the marrow. The observation that also no changes in the cellular compositions of the marrow were found suggests that the sequential flow of cells through granulopoiesis was not disturbed. From these studies it can be concluded that GM-CSF plays an important role in the physiologic regulation of marrow granulocyte release as well as on its production.

One of the characteristics of the granulocyte system is the promptness with which it responds, eg, to infections.¹⁸ The response of this system to rh GM-CSF treatment in monkeys resembles that of an infectious stimulus. Therefore one can speculate that the response of the granulocyte system to an infection is mediated by the release of GM-CSF.

Feedback inhibition of granulopoiesis has long been thought to be essential to the balance that exists between production and utilization.¹⁹ Granulopoiesis simulated by a computer program demonstrated oscillations in production rates.²⁰ This view is supported by the cyclic production of neutrophils in congenital cyclic neutropenia and the appearance of cyclic neutropenia in myelosuppressed dogs.²¹ Mature neutrophils have been shown to inhibit colony formation in vitro²² and to suppress growth of granulocytes in marrow in implanted intraperitoneal diffusion chambers.²³ Substances derived from granulocytes have been reported to inhibit in vitro granulopoiesis.²⁴ The physiologic role of these inhibitors is at present speculative, but it could mean that high numbers of circulating granulocytes can suppress granulopoiesis in vivo. Although granulocyte counts were elevated up to five to six times normal in response to rh GM-CSF treatments, no feedback mechanism suppressing steady-state granulopoiesis and reducing granulocyte counts below normal values were observed after termination of rh GM-CSF treatments in monkeys.

The rise of peripheral blood neutrophil, monocyte, and eosinophil counts in response to the in vivo treatment with rh GM-CSF in monkeys reflects the in vitro observation that this factor also stimulates the proliferation of the corresponding progenitor cells in bone-marrow cell cultures. Although in vitro no lymphocyte colony formation was found in nonhuman-primate bone marrow cultures,¹² in vivo a two-fold to fivefold increase in lymphocyte numbers was observed. This lymphocytosis may represent an indirect effect of rh GM-CSF treatments on hematopoietic regulatory mechanisms. In vitro it was shown that in the presence of erythropoietin rh GM-CSF was capable of stimulating the proliferation of erythroid progenitors in bone marrow cells taken from a nonhuman primate species.¹² It was also suggested that GM-CSF acts directly on erythroid and multipotential progenitor cells, since highly enriched progenitors, depleted of T lymphocytes and monocytes, were also stimulated for colony formation.⁷ Increased reticulocyte counts were also found in control monkeys and can be interpreted as due to repeated blood sampling. Therefore it can be concluded that rh GM-CSF does not have an influence on the erythroid lineage in vivo.

It was particularly interesting that SC treatments (three times daily) were more effective in eliciting higher levels of neutrophils than IV infusions over a six-hour period. Thus the degree of the rh GM-CSF-induced neutrophilia was twofold higher than that obtained after IV infusions. Rh GM-CSF was shown to be cleared from the circulation with a half-life of approximately seven minutes.¹² Also another lymphokine, namely interleukin 2 (IL 2), was cleared from the blood stream rapidly ($t_{1/2}$ five to seven minutes).²⁵ If, however, IL 2 was administered SC in three doses equally throughout the day, IL 2 levels were maintained in the plasma over the entire 24-hour interval. This treatment schedule also showed increased therapeutic activity compared to once-a-day administration.²⁶ It was also found that slowly released IL 2 administered SC from a microosmotic pump was more effective in augmenting therapeutic efficacy than a single injection of the same total dose.²⁷ Therefore the

major factor determining the duration of the biological activity of IL 2 seems to be related to the release of this protein from the site of administration, an observation that may also be due to GM-CSF.

Besides their proliferative effects on committed granulocyte and monocyte progenitor cells, CSFs are also potent stimulators of the functional activity of mature cells. It is, therefore, logical to expect that IV-injected rh GM-CSF would influence the phagocytic activity of mature neutrophils in the circulation. The results of the present study indicate that activation of the peripheral blood neutrophils for increased generation of O_2^- occurred rapidly following the first dose of the CSF (within six hours) and persisted throughout the seven-day dosing regimens. In addition to the enhanced oxidative metabolism, the leukocytes effectively killed a smooth strain of *E coli*.

The dual role of CSF, namely that of the maturation of progenitor cells and the activation of mature cells in the body, has certain attractions. It suggests that the rapid changes in blood CSF levels after certain manipulations, eg, the injection of bacterial endotoxin,²⁸ are primarily to activate mature cells rather than for the stimulation of granulopoiesis.

It was particularly interesting that both the glycosylated mammalian cell-derived and the nonglycosylated *E coli*-derived rh GM-CSF induced a neutrophilia of the same magnitude and duration. The in vivo biological activity of glycosylated and nonglycosylated interferon α was also reported to be the same. Although the interferon carbohydrate side chains were altered by glycosidases, the clearance from the circulation was not changed.²⁹ Also glycosylated and nonglycosylated preparations of IL 2 showed similar biological activities in vivo.³⁰ In contrast, the nonglycosylated form of erythropoietin exerted only weak activity in vivo when compared with the glycosylated form.³¹ It is possible that small glycoproteins like interferon α , IL 2, and GM-CSF having molecular weights around 20 kd are mainly degraded by the kidney,³² whereas larger glycoproteins, eg, erythropoietin with a mol wt of 46 kd, are cleared by the hepatic pathway.³³ This different way of elimination of small and larger glycoproteins may be the reason for variations in the in vivo activity of the glycosylated and nonglycosylated form of some proteins.

The demonstration that rh GM-CSF induces leukocytosis in monkeys suggests a number of potential uses for this lymphokine in humans. For example, GM-CSF could be useful in restoring hematopoiesis in bone marrow transplantation (BMT) patients or in patients experiencing iatrogenic myelosuppression. Because the main function of granulocytes is to protect the body from foreign microorganisms, beneficial therapeutic effects of rh GM-CSF treatment can also be expected against infectious diseases.

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S-100 Beta Positive Human T Lymphocytes: Their Characteristics and Behavior Under Normal and Pathologic Conditions

By Kiyoshi Takahashi, Tadashi Yoshino, Kazuhiko Hayashi, Hiroshi Sonobe, and Yuji Ohtsuki

The characteristics of human S-100 beta-positive T lymphocytes (S-100 beta⁺ T cells) and their fluctuation in peripheral blood under normal and various pathologic conditions were investigated. S-100 beta⁺ T cells were small lymphocytes with no particular subcellular structures and showed a proliferative response to mitogens. They were present mainly in peripheral blood under normal conditions but accumulated in T zones of lymph nodes with nonspe-

cific T-zone hyperplasia, where numerous interdigitating reticulum cells existed. In healthy adults approximately 1% to 4% (mean 3.4%) of peripheral blood mononuclear cells were S-100 beta⁺ T cells. The proportion of S-100 beta⁺ T cells in peripheral blood tended to significantly decrease (<0.5%) in patients with neoplastic diseases; this tendency was apparently related to tumor progression.

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S-100 PROTEINS belong to a family of acidic calcium-binding proteins, such as calmodulin and myosin light chain, whose roles are unknown.^{1,2} Structurally, they are dimers of the alpha and beta subunits, and three distinctive forms, alpha-alpha(S-100a₀), alpha-beta(S-100a), and beta-beta(S-100b) exist.¹⁻⁴ Until recently S-100 proteins were considered to be specific for the nervous system, but recent immunohistochemical studies have revealed that they are widely distributed among human tissues, including lymphoid organs.⁵⁻¹¹ In the human lymphoid organs S-100 beta immunoreactivity is detected in T-zone histiocytes, namely interdigitating reticulum cells (IDC) and Langerhans cells.¹¹ S-100 immunoreactivity has also been detected in some human peripheral blood T cells bearing T-8 antigen.¹² The S-100 positive human T cells show solely S-100 beta subunit immunoreactivity, as demonstrated in the present study. Although the S-100 beta⁺ T cells are present in significant numbers in human peripheral blood, their properties and roles remain unknown. In the present study we attempted to characterize the S-100 beta⁺ T cells with respect to fine structure, mitogen response, membrane properties, and distribution in lymphoid tissues. In addition, to elucidate the role of S-100 beta⁺ T cells, we analyzed and compared their proportions in peripheral blood from healthy controls, from patients with advanced stages of neoplastic diseases, from patients with various stages of gastric cancer, and from patients with nonneoplastic diseases. The results were evaluated in relationship to ages, types, and stages of the diseases.

MATERIALS AND METHODS

Analysis on the Characteristics of Circulating S-100 Beta⁺ T Cells

Preparation of Cells

Peripheral blood mononuclear cells (PBM) were obtained from heparinized peripheral venous blood of healthy adults by Ficoll

Hypaque (FH) density gradient centrifugation, as described previously.¹² T cells were enriched by E rosetting method as described previously.¹² Non-E rosetted-cells were used as a B cell-enriched fraction. T gamma cells (IgG-Fc receptor-positive T cells) were isolated by rosetting technique using IgG-conjugated bovine erythrocytes. Briefly, 1×10^7 cells of T cell fraction were incubated with 5×10^8 bovine erythrocytes conjugated with rabbit IgG (IBL, Takasaki, Japan) for 30 minutes at 37°C. The rosetted cells were separated from nonrosetted cells by FH centrifugation. The erythrocytes in the rosette positive pellet were lysed with 0.85% Tris-NH₄Cl. Complement C3 receptor-positive cells (C3-R⁺ cells) were isolated from PBM by rosetting technique with the use of C3b-conjugated bovine erythrocytes (IBL, Takasaki, Japan) in the same manner. Enriched T cells, enriched B cells, T gamma cells (IgG-Fc-R⁺ T cells), IgG-Fc-R⁻ T cells, C3-R⁺ cells, and C3-R⁻ cells were examined.

Mitogen Stimulation

PBM were cultured in 10% fetal calf serum-supplemented RPMI 1640 containing 10 µg/mL of concanavalin A (con A, Pharmacia, Uppsala, Sweden) or 20 µg/mL of phytohemagglutinin (PHA-M, Gibco, NY) for three days in a CO₂ incubator. The stimulated cells were examined immunocytochemically. The percentage of S-100 beta⁺ cells was compared with that of nontreated PBM cultured for three days without mitogen.

Preparation of Antibodies

Affinity purified rabbit antibodies specific for S-100 alpha or beta subunit were used. The preparation and specificities of these antibodies were described previously.^{10,11} For T cell staining we used monoclonal antibodies (MoAbs), Leu-1 (Becton Dickinson Monoclonal Center Inc, Mountain View, CA), and MT-1 (Bioscience, Switzerland), which recognize all T cells and monocytes but not B cells. To eliminate monocytes that were also positive for MT-1, we used rabbit antilysozyme antisera (DAKO, Denmark), which could specifically detect monocytes. We used MT-1 so that MT-1 could clearly recognize T cells even after formaldehyde fixation, which was essential for detection of the water-soluble S-100 beta subunit. For S-100 and lysozyme staining, we used peroxidase antiperoxidase (PAP) method using goat antirabbit IgG (DAKO, Denmark) and peroxidase antiperoxidase complex (DAKO, Denmark). For Leu-1 and MT-1 staining we used a Vectastain ABC kit (Vector Laboratory Inc, Burlingame, CA).

Immunoperoxidase Staining at the Light Microscopic Level

Cells were fixed in 2% paraformaldehyde solution for five minutes at room temperature, washed with phosphate-buffered saline (PBS), and air dried on glass slides. The cells were immersed in -20°C acetone for ten minutes and incubated in methanol containing 0.3%

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H₂O₂ for 15 minutes to block endogenous peroxidase activity. After washing with PBS the cells were incubated successively with 10% normal goat serum and rabbit antibodies against either S-100 alpha or S-100 beta subunit (15 µg/mL) for 60 minutes. After washing with PBS the cells were stained by PAP method, as described previously.¹² For Leu-1 or MT-1 staining, cells treated with normal goat serum were incubated with MoAb Leu-1 (1:20) or MT-1 (1:10) and stained by the ABC method according to the staining procedure of a Vectastain ABC kit.

Immunoperoxidase Staining at the Electron Microscopic Level

Affinity-purified antibody to S-100 beta subunit was conjugated with horseradish peroxidase (HRPO) by the method of Nakane and Kawaoi.¹³ Enriched T cells were fixed, stained with the HRPO-conjugated anti-S-100 beta, embedded in Epon 812, and examined as described previously.¹²

Analysis of Morphology and Distribution of S-100 Beta⁺ T Cells in Human Lymphoid Tissues

Preparation of Lymphoid Tissues

Each of the five formalin-fixed specimens of normal lymph nodes, thymus, and spleen was obtained at autopsy. Fifteen lymph nodes with nonspecific lymphadenitis showing marked T-zone hyperplasia were obtained by surgical biopsy. Tissues were routinely fixed in formalin and embedded in paraffin.

Immunoperoxidase Staining of Tissues at Light Microscopic Level

Deparaffinized sections were stained with rabbit anti-S-100 beta antibody by PAP method as described previously.¹⁰

Immunoelectron Microscopic Analysis

Slices 60-µm thick were cut from formalin-fixed specimens by an Oxford Vibratome. The slices were stained with anti-S-100 beta by indirect immunoperoxidase method using HRPO-conjugated goat antirabbit IgG (DAKO, Denmark), as described in our previous paper.⁸

Analysis of the Fluctuation of Circulating S-100 Beta⁺ T Cells Under Normal and Pathologic Conditions

Selection of Patients and Controls

Controls. Healthy adult donors consisted of 38 individuals 20 to 92 years of age, 24 males and 14 females. Of 38 healthy individuals, 24 persons were under 60 years of age (mean = 30 years, younger age group) and 14 persons were over 60 years of age (mean = 76 years, older age group).

Patients with advanced neoplastic diseases. Patients of this group had a dissemination or systemic metastasis of the neoplasm. They consisted of 49 individuals 22 to 88 years of age, 25 males and 24 females, including 15 patients with lung cancer (six adenocarcinomas, five squamous cell carcinomas, four small cell carcinomas), 13 patients with gastric carcinoma, seven patients with non-Hodgkin malignant lymphoma, three patients each with gallbladder carcinoma and mammary carcinoma, two cases with hepatocellular carcinoma, and single cases with carcinomas of the thyroid, pancreas, rectum, renal clear cells, esophagus, and larynx.

Patients with early-stage gastric cancer. Patients of this group had a gastric adenocarcinoma restricted to gastric mucosa and showed neither clinical symptoms nor abnormalities in laboratory

tests. The patients consisted of seven individuals 46 to 70 years of age, three males and four females.

Patients with a postoperative status of gastric cancer. Patients in this group had received surgical therapy over 2 years before lymphocyte analysis and had a curative status without evidence of metastasis or recurrence. The patients consisted of 16 individuals 30 to 80 years of age, nine males and seven females.

Patients with non-neoplastic diseases. Patients consisted of 39 individuals 24 to 81 years of age, 25 males and 14 females, including eight patients with diabetes mellitus; four patients each with chronic active hepatitis, liver cirrhosis, and nephrotic syndrome; three patients each with lower respiratory disease and hypertension; two patients each with systemic lupus erythematosus (SLE) and chronic thyroiditis; and individual patients with chronic pancreatitis, gastroenteritis, polymyositis, progressive systemic sclerosis (PSS), sialoadenitis, splenic infarction, primary myocardopathy, necrotizing lymphadenitis, and cerebrovascular disease. None of the patients received chemotherapy or radiotherapy during the month before lymphocyte analysis. The patients with SLE, nephrotic syndrome, polymyositis, and PSS received extensive steroid therapy.

Analysis of the Percentages of Circulating S-100 Beta⁺ T Cells and Total T Cells

PBM were obtained from 5 mL of heparinized peripheral venous blood of the healthy controls and patients as described above. PBM of all persons were stained with anti-S-100 beta as described above. Besides S-100 beta staining, PBM from patients with gastric cancer, including 13 advanced cases, seven early cases, and 16 cases with postoperative stages, were stained with MT-1 and lysozyme to analyze the proportions of total T cells. Both S-100 beta-positive and negative cells were precisely counted by tracing these cells in pictures of nonselected fields until they reached 1,000 in number, and the percentage of the positive cells was calculated. After four counts the average percentage was used as the result. The percentages of total T cells were calculated by counting MT-1 positive cells after eliminating lysozyme-positive monocytes.

RESULTS

Characteristics of Circulating S-100 Beta⁺ T Cells

S-100 Subunit Immunoreactivities

S-100 beta⁺ cells were detected exclusively in the T cell-enriched fraction. Approximately 4% of T cells were positive for the S-100 beta subunit (Fig 1A). Almost all cells of this fraction were positive for Leu-1, but none was positive for S-100 alpha subunit. No S-100 beta⁺ cells were detected in the B cell-enriched fraction. Monocytes in the B-enriched fraction were clearly positive for S-100 alpha. These findings indicated that approximately 4% of the human peripheral T cells contained S-100b protein, a dimer of S-100 beta subunit. Therefore we named them S-100 beta⁺ T cells.

Response to Mitogens

The majority of PBM from healthy donors treated with con A or PHA-M for three days displayed a marked blastic change, and some of the blastic cells were clearly positive for S-100 beta. Occasionally S-100 beta immunostaining was found in mitotic cells (Fig 1B). The percentages of S-100 beta positive cells in con A- or PHA-treated PBM were 3.5 or 3.4 respectively and were almost equivalent to that in non-treated PBM (3.3%). These findings indicated that S-100 beta⁺ T cells had an ability to display a proliferative response

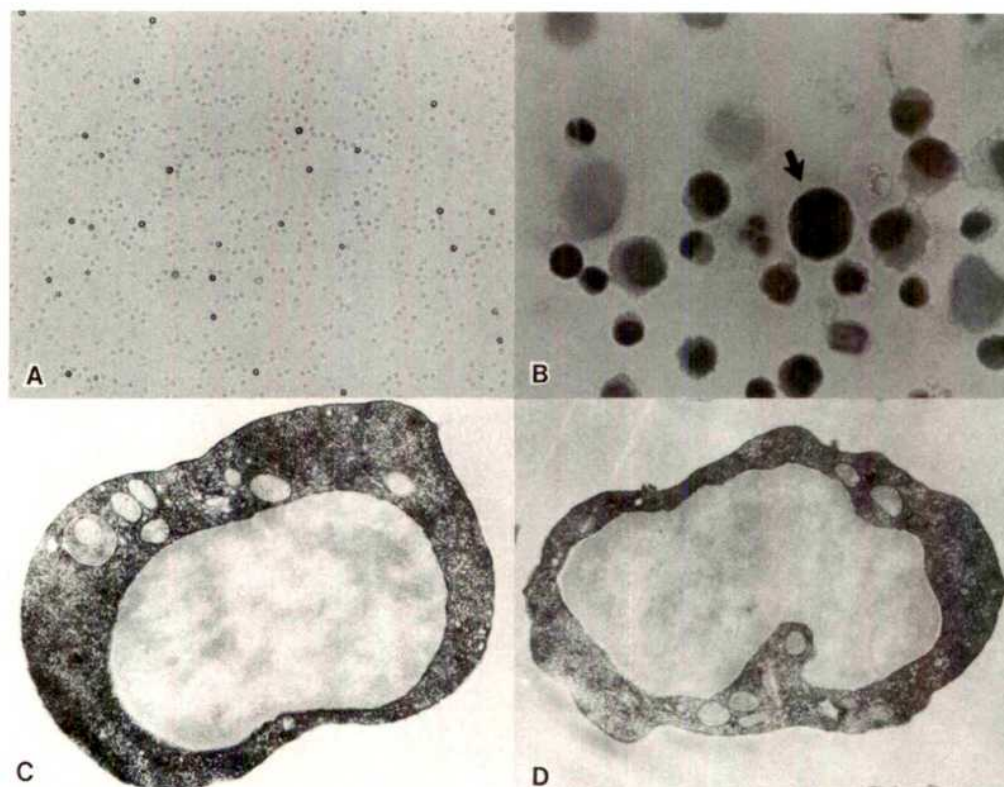


Fig 1. (A) S-100 beta immunoperoxidase staining of T cell-enriched fraction of healthy donors. Approximately 4.0% of T cells of peripheral blood are S-100 beta⁺ T cells (original magnification $\times 80$; current magnification $\times 60$). (B) S-100 beta-immunoperoxidase staining of peripheral blood mononuclear cells stimulated by con A for three days. Note a mitotic cell showing intense S-100 beta immunoreactivity (arrow) (original magnification $\times 1,000$; current magnification $\times 750$). (C, D) Immunoelectron micrographs of S-100 beta⁺ T cells in peripheral blood of healthy donor. S-100 beta⁺ T cells possess narrow cytoplasm with scanty cellular organelles and round (A) or singly indented (B) nuclei (original magnification $\times 9,300$; current magnification $\times 6,975$).

to con A and PHA, and the S-100b protein in the cytoplasm was stable during the proliferative reactions.

IgG-Fc Receptor

S-100 beta immunoreactivity was detected in approximately 5% of T gamma cells. S-100 beta immunoreactivity was also detected in about 3% of IgG-Fc-R⁻ cells. Of 1×10^7 T cells, approximately 20% were IgG-Fc-R positive (T gamma cells), and 80% were IgG-Fc-R negative. From these findings it was estimated that about 30% of S-100 beta⁺ T cells expressed IgG-Fc receptor, but the rest did not.

Complement C3 Receptor

No S-100 beta⁺ T cells were detected in C3-R⁺ fraction. S-100 beta⁺ T cells were found exclusively in C3-R⁻ fraction. These findings indicated that S-100 beta⁺ T cells did not express complement C3 receptor.

Fine Structure of Circulating S-100 Beta⁺ T Cells

Ultrastructurally S-100 beta⁺ T cells in peripheral blood were small, smooth-surfaced lymphocytes containing round or singly indented nuclei and had narrow cytoplasm with scanty cellular organelles (Figs 1C, 1D). Immunoreaction products were evenly distributed throughout the cytoplasm except for mitochondria and vacuoles. Usually nuclei were devoid of the reaction products.

Morphology and Distribution of S-100 Beta⁺ T Cells in Human Lymphoid Tissues

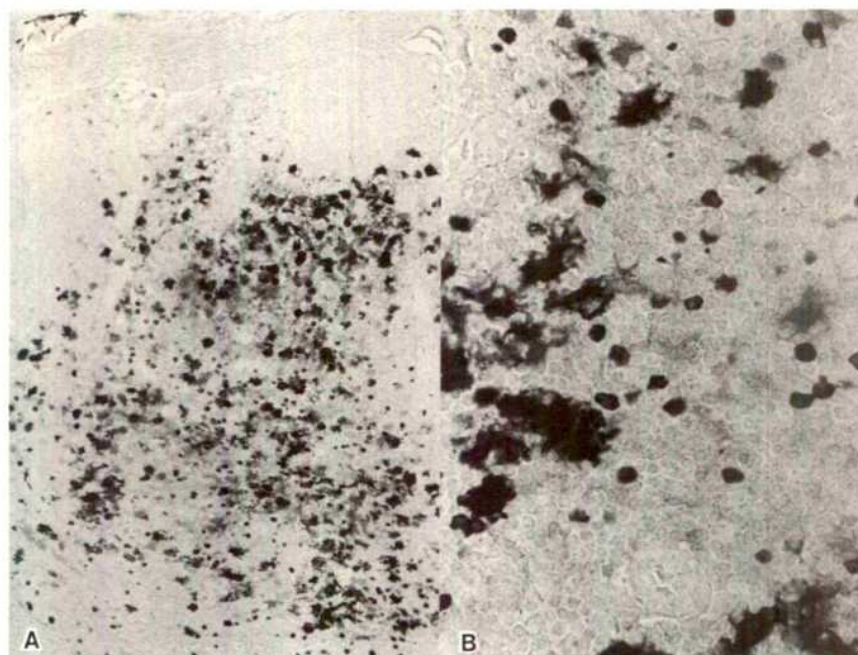
Immunohistochemistry

In each case of normal lymph node, thymus, and spleen, only a small number of S-100 beta⁺ T cells were detected in T zones. Thus under normal conditions S-100 beta⁺ T cells circulated mainly in the peripheral blood. However, relatively large numbers of S-100 beta⁺ T cells were found in lymph nodes with reactive T-zone hyperplasia, and these cells were found exclusively in T zones where numerous S-100 beta⁺ IDC were present (Fig 2A). In addition, it was of great interest that a variety of S-100 beta⁺ cells showing morphological transition from lymphocytes to IDC were frequently observed in these areas (Fig 2B).

Immunoelectron Microscopy

S-100 beta immunoreactivity was detected in IDC and small lymphocytes (Figs 3A, 3C). As shown in our previous study,⁹ S-100 beta⁺ IDC possessed considerable cytoplasm with interdigitating projections and irregularly shaped nuclei with evenly distributed nuclear chromatin (Fig 3C). S-100 beta⁺ T cells in lymph nodes were small lymphocytes with singly indented nuclei and narrow cytoplasm without characteristic subcellular structure (Fig 3A). Occasionally small lymphocyte-sized or intermediate-sized cells that possessed

Fig 2. S-100 beta immunoperoxidase staining of a lymph node with reactive T-zone hyperplasia. (A) Note numerous S-100 beta⁺ T cells in T zone where large numbers of S-100 beta-positive interdigitating reticulum cells are present (original magnification $\times 100$; current magnification $\times 70$). (B) A number of S-100 beta-positive cells showing morphological transition from lymphocytes to S-100 beta-positive interdigitating reticulum cells (original magnification $\times 400$; current magnification $\times 280$).



round or irregular nuclei and interdigitating cytoplasmic projections were found to be positive for S-100 beta subunit (Fig 3B).

The Fluctuation of S-100 Beta⁺ T Cells Under Normal or Pathologic Conditions

Healthy Controls

The percentages of circulating S-100 beta⁺ T cells in the peripheral blood of 38 healthy controls ranged from 0.5 to 10.0, and the mean was 3.4% (Fig 4). No sexual difference was detected. As for ages, the mean of the older age group (over 60 years of age) was 2.9%, and that of the younger age group (under 60 years of age) was 3.6%. The former was statistically lower than the latter (*t* test, $P < 0.05$). Of 38 healthy controls, only four persons showed less than 1.0%, but none of them showed less than 0.5%. Thus we concluded

that a percentage lower than 0.5 indicated an abnormally low proportion of S-100 beta⁺ T cells in peripheral blood.

Patients With Advanced Stages of Neoplastic Diseases

In almost half of the patients (24 of 49), the ranges for S-100 beta⁺ T cells were less than 0.5% (Fig 4). Of these 24 patients, 18 subjects showed less than 0.2%. About two thirds of subjects showed less than 1.0%. The mean was 0.9%, and the lowest percentage was 0.035, which was observed in a patient with gastric cancer. A marked decrease in S-100 beta⁺ T cells was observed in both young and old individuals with advanced cancer but was not correlated with the ages as shown in Fig 4. As for types of the diseases, a percentage of S-100 beta⁺ T cells lower than 0.5 was observed in eight of 13 patients with gastric cancer, in seven of 15 patients with lung cancer (four of six cases with adenocarcinoma, two of four

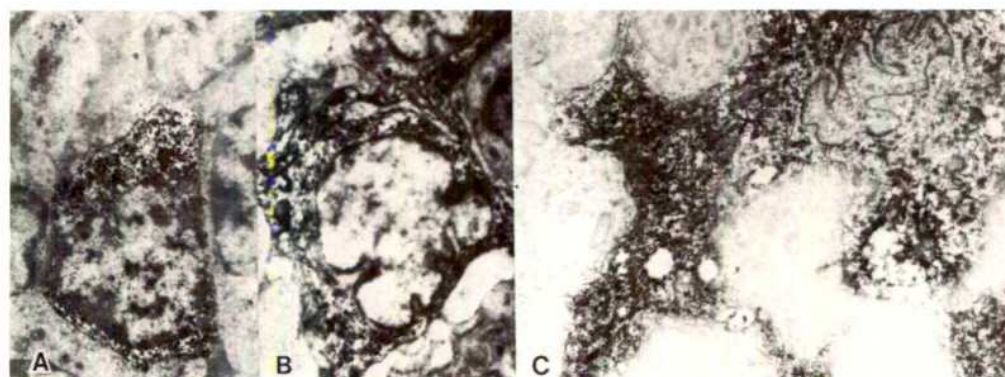


Fig 3. Immunoelectron micrographs of S-100 beta-positive cells in a lymph node with reactive T-zone hyperplasia. (A) A small lymphocyte showing S-100 beta immunoreactivity. The lymphocyte contains a singly indented nucleus and narrow cytoplasm with scanty cellular organelles (original magnification $\times 6,000$; current magnification $\times 4,500$). (B) S-100 beta-positive cells of lymphocyte-size with irregular nucleus and interdigitating cytoplasmic processes (original magnification $\times 5,000$; current magnification $\times 3,750$). (C) Interdigitating reticulum cells showing S-100 beta immunoreactivity. The cell contains an irregular nucleus with evenly distributed nuclear chromatin and possesses wide cytoplasm showing abundant cellular organelles and dendritic cytoplasmic projections (original magnification $\times 3,300$; current magnification $\times 2,475$).

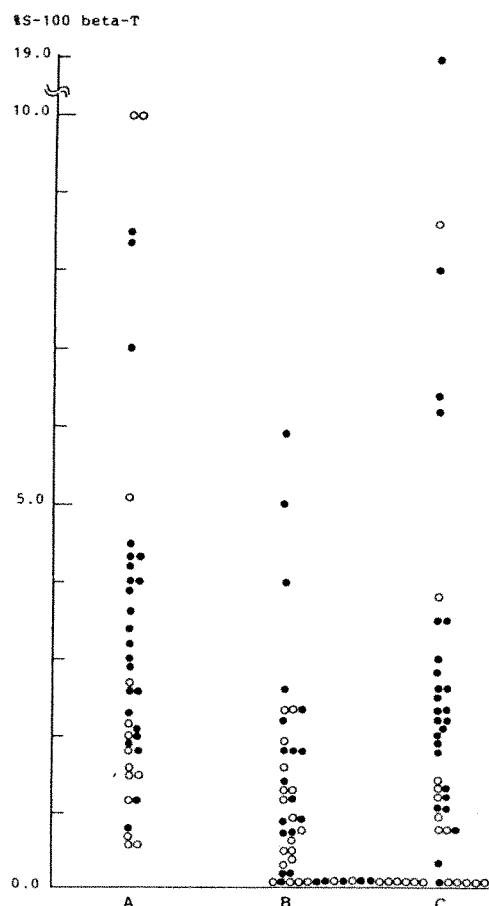


Fig 4. The percentage of S-100 beta⁺ T cells in peripheral blood from healthy controls (A), patients with advanced neoplastic diseases (B), and patients with non-neoplastic diseases (C). ●, individual under 60 years of age; ○, individual over 60 years of age.

patients with squamous cell carcinoma, and one of four patients with small cell carcinoma), in three of seven patients with non-Hodgkin lymphoma, in two of three patients with mammary carcinoma, in one of two patients with hepatocellular carcinoma, and in one of three patients with gallbladder carcinoma, and in single patients each with esophageal carcinoma and laryngeal carcinoma.

Patients With Early or Postoperative Status of Gastric Cancer

In contrast to the patients with advanced gastric cancer, only one of seven patients with early gastric cancer and none of 16 patients with postoperative status of gastric cancer ranged less than 0.5%. No statistical difference in the percentage of S-100 beta⁺ T cells was noticed between these groups and healthy controls (Fig 5).

Patients With Non-neoplastic Diseases

Of 39 individuals, seven persons showed less than 0.5%, including two of eight patients with diabetes mellitus, two of four patients with chronic active hepatitis, two of two patients with SLE, and one of three patients with hyperten-

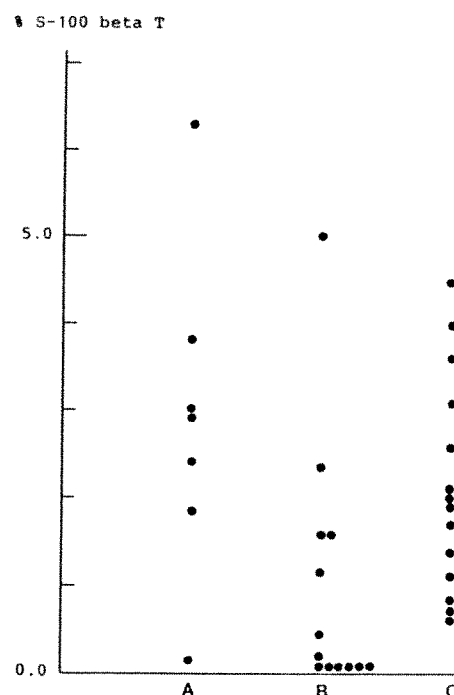


Fig 5. The percentage of S-100 beta⁺ T cells in peripheral blood of patients with early stage gastric cancer (A), patients with advanced stages of gastric cancer (B), and patients with a curative status of postoperative gastric cancer (C).

sion. In contrast to patients with advanced neoplastic diseases, the majority of this group (29 out of 39) showed more than 1.0% (mean = 2.3%). The highest percentage was 19.0, observed in patients with nephrotic syndrome (Fig 5A).

The Proportions of Total T Cells in Patients With Gastric Cancer

The percentages of total T cells recognized by MT-1 in peripheral blood of these groups ranged from 60 to 85, and no statistical difference was detected among these three groups.

DISCUSSION

Previously we reported that the S-100 positive T lymphocytes expressed T-3, T-8, and T-11 antigens but not T-4, HLA-DR, Leu-7, or OKM1 antigens.¹² In the present study we further characterized the S-100 positive human T lymphocytes. They showed solely S-100 beta subunit immunoreactivity and ultrastructurally were small lymphocytes with no characteristic subcellular structure; they displayed a proliferative response to con A and PHA. These findings further indicated the distinct T cell nature of S-100 beta⁺ T cells. The S-100 beta⁺ T cells were present mainly in peripheral blood under normal conditions. Approximately 3% to 4% of peripheral blood mononuclear cells were S-100 beta⁺ T cells in healthy persons. We also found that the proportion of circulating S-100 beta⁺ T cells of healthy persons displayed a tendency to decrease with age. In contrast, a markedly decreased percentage of S-100 beta⁺ T cells (less than 0.5%) was observed in nearly 50% of patients

with advanced neoplastic diseases. The marked decrease in the percentage of S-100 beta⁺ T cells was not significantly related to age or histologic types of tumors. The present analysis of the patients with various stages of gastric cancer also revealed that such a decrease was apparently related to the advanced stage of the disease. As for T cell profiles, no significant difference in total T cell proportion was observed among the patients with various stages of gastric cancer. Although S-100 beta⁺ T cells have been shown to belong to the T-8⁺ cell subpopulation,¹² there has, as far as we know, been no report that indicates the proportional decrease of T-8⁺ cells in cancer patients accompanied by tumor progression, which could account for such a marked proportional decrease of S-100 beta⁺ T cells. Ginn et al¹⁴ reported that in the patients with primary lung cancer, no significant difference in the proportion of total T cells (T-3⁺), helper/inducer T cells (T-4⁺), or suppressor/cytotoxic T cells (T-8⁺) was detected as compared with healthy controls, although their absolute numbers differed according to the histologic types of the tumors. It has been shown that the proportion of T gamma cells may increase in the patients with advanced cancer.^{15,16} We found, however, that the vast majority of T gamma cells was negative for S-100 beta. Thus it was likely that the proportional fluctuation of S-100 beta⁺ T cells was independent of those of other types of T cells. Proportional independence of S-100 beta⁺ T cells from other T cell subsets suggests that their roles are distinct from those of other T cell subsets. It is also possible that such a marked proportional decrease of S-100 beta⁺ T cells resulted in or from certain immunologic abnormalities in patients with advanced neoplasms. It has been well documented that cancer patients may display various immunodeficiency disorders correlating with stages of disease,¹⁷⁻²¹ which have generally been considered to be important for tumor cells in escaping from the host's immunologic surveillance system. Therefore it is possible that a marked proportional decrease in S-100 beta⁺ T cells may be linked to known or unknown immunodeficiency disorders in the patients with advanced cancer.

In this study we found that S-100 beta⁺ T cells circulated mainly in peripheral blood under normal conditions, but in certain pathologic conditions they accumulated in T zones of lymphoid tissue where numerous IDC were usually present. Moreover a considerable number of S-100 beta-positive cells of various intermediate sizes between lymphocytes and IDC were detected in these areas. They seemed to be in the course of conversion from lymphocytes into IDC. Such coexistence of S-100 positive, small, lymphocyte-like cells and intermediate cells with IDC in T zones was also noticed and reported by Watanabe et al.²² They regarded the S-100 positive, small, lymphocyte-like cells and intermediate cells as precursors of IDC but did not recognize S-100 positive, small, lymphocyte-like cells as T lymphocytes. However, the present immunoelectron microscopic findings clearly indicated that they possessed the distinct morphological features of small lymphocytes. We also detected IL2 receptor on con A-stimulated S-100 beta⁺ T cells (unpublished finding). As shown in our previous¹² and present studies, the S-100 positive, lymphocyte-like cells definitely are T cells, and they form a novel human T cell subset, namely, the S-100 beta-positive T cell subset. Thus the question as to whether S-100 beta⁺ T cells are precursors of IDC and their related cells should be settled because the origin of IDC still remains undetermined. There are, however, extensive differences between S-100 beta⁺ T cells and IDC. Namely, IDC and its related cells have been shown to possess HLA-DR, T-6 antigens, complement receptor, ATPase activity, none of which has been proved in S-100 beta⁺ T cells.^{12,23} IDC have generally been considered and accepted to be antigen-presenting cells for T cells but not to be cells originating from T lymphocytes.²⁴ At present we have no evidence to support the view that S-100 beta⁺ T cells can convert into IDC, except for the immunomorphological findings. Further studies should be made to resolve these questions.

Recently Ruco et al²⁵ reported an unusual case of lymph node neoplasm in which tumor cells were positive for S-100 protein and expressed sheep erythrocyte receptor, T-3, T-8, and T-11 antigens. According to their observations, the neoplastic cells with S-100 immunoreactivity undoubtedly were T cells. Thus their particular case of lymph node neoplasm implies the presence in a neoplastic disorder of S-100 beta⁺ T cells. In fact, we found a patient with a neoplastic proliferation of S-100 beta⁺ T cells in peripheral blood (unpublished data). Therefore we are considering proposing a new entity of T cell malignancy, namely S-100 beta⁺ T cell leukemia/lymphoma.

Recently, Nomori et al²⁶ suggested the important role of S-100 positive T-zone histiocytes, IDC, and Langerhans cells in antitumor immunity of humans. They found that the degree of the density of S-100 positive T-zone histiocytes, but not that of ordinary macrophages, in the primary site of nasopharyngeal carcinoma had a significant relationship to prognosis. Together with our findings it is more likely that S-100 beta-positive cells in the human immune system may play important roles in antitumor immunity.

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Fibrinogen-Independent Aggregation and Deaggregation of Human Platelets: Studies in Two Afibrinogenemic Patients

By M. Cattaneo, R.L. Kinlough-Rathbone, A. Lecchi, C. Bevilacqua, M.A. Packham, and J.F. Mustard

Platelets from two afibrinogenemic patients were used to determine whether fibrinogen is essential for platelet aggregation and to examine whether released fibrinogen contributes to the stabilization of platelet aggregates when platelets have been induced to aggregate and release their granule contents by stimulation with thrombin. The addition of adenosine diphosphate (ADP) to platelet-rich plasma (PRP) or to suspensions of washed platelets from the afibrinogenemic patients caused the formation of small aggregates, which was either not inhibited or only slightly inhibited by the F(ab')₂ fragments of an antibody to fibrinogen but was inhibited by an antibody (10E5) to glycoprotein IIb/IIIa. Thus there is a component of ADP-induced platelet aggregation that is not dependent on fibrinogen or other plasma proteins but is dependent on glycoprotein IIb/IIIa.

FIBRINOGEN is believed to be important in human platelet aggregation because platelets from patients with afibrinogenemia aggregate poorly in response to adenosine diphosphate (ADP),¹⁻⁴ and washed human platelets also aggregate poorly in response to ADP if they are suspended in a medium without added fibrinogen.⁵⁻¹² In addition, thrombasthenic platelets that lack the fibrinogen receptor glycoprotein IIb/IIIa do not aggregate in response to any agonist,¹³⁻²⁰ and antibodies directed against glycoprotein IIb/IIIa inhibit platelet aggregation and the binding of fibrinogen.²¹⁻²⁵ However, despite these observations it is not known whether fibrinogen is essential for platelet aggregation. Several observations indicate that a small extent of ADP-induced aggregation occurs without the addition of fibrinogen to suspensions of washed human platelets in a medium containing 2 mmol/L Ca²⁺ and 1 mmol/L Mg²⁺, in which ADP does not cause appreciable release of platelet granule contents,^{1,12,26} and in which suspensions of washed platelets from rabbits aggregate extensively in response to ADP without the addition of fibrinogen to their suspending medium.^{27,28}

When aggregation is induced by agents that cause the release of granule contents, released fibrinogen is thought to be responsible for aggregation. Tollefsen and Majerus²⁹ reported that Fab fragments of an antibody to fibrinogen inhibited thrombin-induced aggregation. However, although Legrand et al.³⁰ also found that Fab fragments of an antibody to fibrinogen were able to partially inhibit platelet aggregation caused by very low concentrations of thrombin, high concentrations were not inhibited. Further evidence that aggregation can occur without fibrinogen includes the observations of Turitto et al.,³¹ who have shown that platelets from the native blood of an afibrinogenemic patient form large aggregates in a Baumgartner flow chamber, and the studies of Soria et al.,¹ showing that washed platelets from afibrinogenemic subjects aggregate normally in response to thrombin.

It has not been established whether fibrinogen plays a major role in preventing platelet deaggregation. When platelets aggregate without releasing granule contents, deaggregation occurs readily, and the fibrinogen that has been bound

There was little difference in the extent of aggregation and the release of granule contents of normal and afibrinogenemic platelets in response to the release-inducing agents collagen, platelet-activating factor (PAF), sodium arachidonate, or thrombin. With normal or afibrinogenemic platelets, aggregation by thrombin (0.2 U/mL or higher) was not inhibited by the F(ab')₂ fragments of an antibody to human fibrinogen. Deaggregation by combinations of inhibitors of platelets aggregated by 1 U/mL thrombin showed no difference between platelets from afibrinogenemic and control subjects, indicating that released fibrinogen does not make a major contribution to the stabilization of platelet aggregates formed by thrombin stimulation.

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during aggregation readily dissociates.^{12,32,33} In contrast, when the release of granule contents takes place, platelets do not deaggregate readily, and even when they are induced to deaggregate, only some of the bound fibrinogen dissociates.³⁴

Platelets from afibrinogenemic patients provided an opportunity to determine whether fibrinogen is essential for platelet aggregation and to examine the contribution of released fibrinogen to the stabilization of thrombin-induced platelet aggregates.

MATERIALS AND METHODS

Materials. ¹⁴C-serotonin (¹⁴C-5-hydroxytryptamine-3'-creatinine sulfate, 50 mCi/mmol) was from Amersham Corporation, Arlington Heights, IL. Bovine serum albumin, Pentex fraction V, was from Miles Laboratories, Elkhart, IN. Apyrase was prepared from potatoes by the method of Molnar and Lorand.³⁵ The activity of this preparation in the final platelet-suspending medium was such that the enzyme (1 μL/mL) converted 0.25 μmol/L adenosine triphosphate (ATP) to adenosine monophosphate (AMP) within 120 seconds at 37°C. Human fibrinogen (grade L, AB Kabi, Stockholm, Sweden) was pretreated with diisopropylfluorophosphate (DFP, Sigma Chemical Co, St. Louis)³⁶ and was partially purified by the method of Lawrie et al.³⁷ Chymotrypsin, ADP, collagen, and sodium arachidonate were from Sigma. Heparin was from Pentapharma (Basel, Switzerland). Synthetic platelet-activating factor (PAF,

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1-O-octadecyl-2-acetyl-sn-glycerol-3-phosphoryl-choline) was from Bachem Feinchemikalien (Bubendorf, Switzerland). Thrombin was from Miles and PGE₁ from the Upjohn Company (Kalamazoo, MI). A monoclonal antibody (MoAb) to GPIIb/IIIa (10E5) was kindly provided by Dr Barry Coller (Stony Brook, NY) and was dialyzed against 0.14 mol/L NaCl solution before use. F(ab')₂ fragments of an antibody to human fibrinogen raised in goats were from Cappel (Malvern, PA); F(ab')₂ fragments of nonimmune IgG were from the same source. Sodium arachidonate and PGE₁ were prepared as previously described.^{38,39} Acid-soluble collagen was prepared by the method of Cazenave et al.⁴⁰ All other solutions were dissolved in modified Tyrode's solution. Citrated platelet-rich plasma (PRP) and suspensions of washed human platelets were prepared as described previously.^{11,41}

Aggregation and release. Platelet aggregation and the release of ¹⁴C-serotonin from prelabeled platelets was measured as described previously.^{41,42} Release of ATP from platelets was measured in a lumi-aggregometer (Chronolog, Haverton, PA).

Platelet count. The platelet count in PRP was adjusted to 350,000/ μ L; in suspensions of washed platelets it was 500,000/ μ L.

Fibrinogen assay. Platelet and plasma fibrinogen were measured using a fibrinopeptide A assay (FPA) kit (Mallinckrodt, St. Louis). FPA was freed from the fibrinogen by incubation with thrombin (4 U/mL) for two hours at 37°C. For measurement of platelet fibrinogen, platelets were treated with Triton X100, and the lysate was incubated with thrombin.

Characteristics of patients. Both patients with afibrinogenemia were young females (18 and 19 years of age) with a history of bleeding episodes dating from early childhood. Their bleeding times were prolonged (12 minutes and 18 minutes respectively, control 2.5 to seven minutes) and both the prothrombin times and partial thromboplastin times were greater than 600 seconds (controls 13 seconds and 28 seconds respectively). Neither patient had detectable amounts of plasma fibrinogen, as measured by the fibrin polymerization time (Bonomelli, Milan, Italy).

RESULTS

Fibrinogen concentrations. The concentrations of fibrinogen in the platelets of the afibrinogenemics and normal subjects are shown in Table 1. Both patients had plasma fibrinogen concentrations of approximately 3 μ g/mL.

Citrated PRP. The addition of ADP to citrated PRP prepared from the blood of the two afibrinogenemic patients caused the formation of small platelet aggregates; the addition of fibrinogen enhanced the response (Fig 1). Collagen, PAF, or sodium arachidonate induced more extensive aggregation of afibrinogenemic platelets, although the extent of aggregation was somewhat less than in PRP prepared from normal subjects (Fig 2). Aggregation of the afibrinogenemic platelets in response to arachidonate occurred after a lag phase, whereas normal platelets aggregated more rapidly. The amount of ATP released in response to the agonists was similar to or less than the amount released from normal platelets under the conditions used (Fig 2).

Table 1. Platelet Fibrinogen Concentrations in Patients With Afibrinogenemia and in Control Subjects

Subjects	n	Platelet Fibrinogen μ g/ 10^9 Platelets
Patient I	1	14.2
Patient II	3	8.6 $\pm$ 2.1
Controls	9	312.0 $\pm$ 17.0

Mean values $\pm$ SEM.

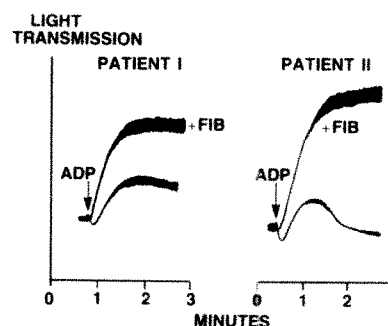


Fig 1. Platelet aggregation induced by ADP (2 μ mol/L) in citrated PRP obtained from the blood of two patients with afibrinogenemia. The upper tracings show the enhancement of aggregation induced by fibrinogen (FIB; 400 μ g/mL) added 30 seconds before the ADP. Platelet count: 350,000/ μ L.

To investigate the possible contribution to the extent of ADP-induced aggregation of the small amount of fibrinogen in afibrinogenemic plasma, the effect of F(ab')₂ fragments of an antibody to human fibrinogen was investigated. There was no inhibitory effect on aggregation induced by 2 μ mol/L ADP in comparison with control samples to which nonimmune F(ab')₂ fragments had been added; aggregation curves with the nonimmune fragments were superimposable on aggregation curves to which no F(ab')₂ fragments were added (Fig 3A). Addition of 8 μ g/mL of fibrinogen to afibrinogenemic PRP enhanced aggregation to some extent, and this enhancement was eliminated by the F(ab')₂ fragments of the antibody to fibrinogen (Fig 3B). Again, the aggregation curve obtained with nonimmune control F(ab')₂ fragments was superimposable on the aggregation curve obtained with only 8 μ g/mL fibrinogen added before the ADP.

Suspensions of washed platelets. For these experiments the platelets were resuspended in Tyrode-albumin solution, which is a balanced salt solution containing 2 mmol/L

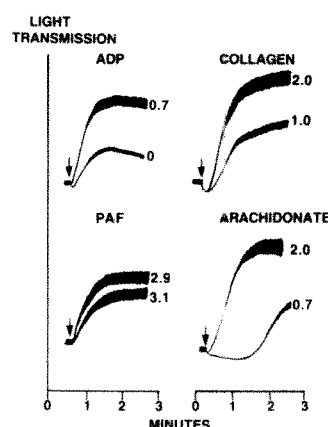


Fig 2. Platelet aggregation induced by ADP (2 μ mol/L), collagen (2 μ g/mL), PAF (0.25 μ mol/L), or sodium arachidonate (1 mmol/L) in citrated PRP prepared from the blood of an afibrinogenemic patient (the lower tracing in each panel) and a normal control (the upper tracing). The amount of ATP released (μ mol/L) is indicated beside the aggregation tracings. Platelet count: 350,000/ μ L. Results shown are for patient II, but similar results were obtained with patient I.

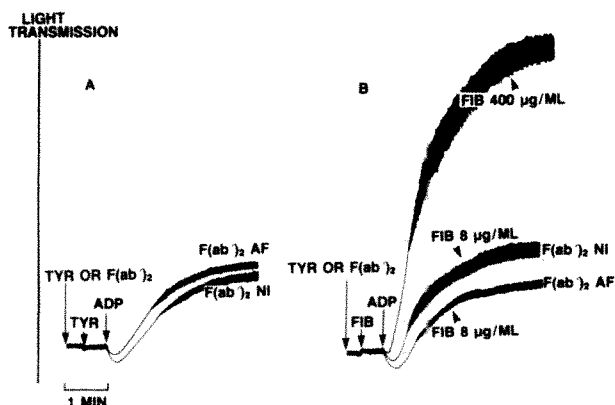


Fig 3. Platelet aggregation induced by ADP (2 $\mu\text{mol/L}$) in citrated PRP obtained from patient II. (A) Comparison of the effect of F(ab')_2 fragments of an antibody to fibrinogen (F(ab')_2 AF; 220 $\mu\text{g/mL}$) with F(ab')_2 fragments of nonimmune IgG (F(ab')_2 NI; 250 $\mu\text{g/mL}$). The aggregation curve obtained without the addition of F(ab')_2 fragments was superimposable on the F(ab')_2 NI curve. Tyrode's solution (TYR) was added to maintain constant volumes. (B) Similar to (A) except fibrinogen (FIB) 8 $\mu\text{g/mL}$ or 400 $\mu\text{g/mL}$ was added before ADP. Again, the aggregation curve obtained without the addition of F(ab')_2 fragments was superimposable on the F(ab')_2 NI curve. Platelet count: 400,000/ μL .

calcium, 1 mmol/L magnesium, 0.35% albumin and apyrase. In this medium ADP in the presence of fibrinogen causes only the primary phase of aggregation of normal platelets, regardless of the concentration of ADP used^{26,41}; thromboxane formation does not occur, and there is little or no release of the contents of the platelet storage granules.⁴³

Figure 4 shows that ADP caused washed platelets from both an afibrinogenemic patient and from a normal subject to aggregate to a small extent. Microscopic examination confirmed that some of the platelets were present as small aggregates. The addition of fibrinogen before ADP enhanced the aggregation response and resulted in the formation of large aggregates (Fig 4). In all cases aggregation was followed by deaggregation.

With both the normal and afibrinogenemic platelets the small amount of aggregation that was observed in the absence of fibrinogen was either not inhibited or only slightly inhibited by the F(ab')_2 fragments of an antibody to fibrino-

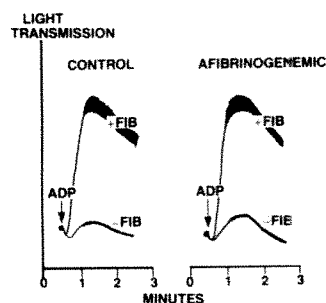


Fig 4. Platelet aggregation induced by ADP (20 $\mu\text{mol/L}$) added to suspensions of washed platelets in a medium containing 2 mmol/L Ca^{2+} , 1 mmol/L Mg^{2+} , albumin (0.35%), and apyrase. The upper tracings show the enhancement of aggregation caused by fibrinogen (FIB; 400 $\mu\text{g/mL}$) added 30 seconds before the ADP (Patient II).

gen that completely inhibited the potentiation of aggregation caused by the addition of fibrinogen (3 to 11 $\mu\text{g/mL}$) (Fig 5). (Control experiments were done with nonimmune F(ab')_2 fragments.) However, an antibody to glycoprotein IIb/IIIa (10E5) at a final concentration of 10 $\mu\text{g/mL}$ completely inhibited platelet aggregation induced by 10 to 20 $\mu\text{mol/L}$ ADP but had no effect on the shape change of the platelets.

There was little difference in the extent of aggregation and the release of granule contents of normal and afibrinogenemic platelets in response to thrombin (0.2 to 1.0 U/mL; Fig 6). In these experiments the addition of hirudin two minutes after thrombin did not lead to deaggregation of the platelets from either the normal or afibrinogenemic patient (data not shown). Aggregation by thrombin at a concentration of 0.2 U/mL or higher was not inhibited by the F(ab')_2 fragments of an antibody to human fibrinogen (Fig 6).

Deaggregation. To investigate the possible role of fibrinogen in preventing deaggregation, we examined the effect of several combinations of inhibitors that we have previously shown cause the deaggregation of human platelets aggregated by thrombin.⁴⁴ Figure 7 shows the effect of the combination of hirudin, EDTA, and chymotrypsin two minutes after the addition of thrombin to suspensions of washed platelets. The rate and extent of deaggregation was practically identical for the normal and afibrinogenemic platelets. Inhibitors that did not deaggregate either type of platelet in the presence of hirudin were PGE_1 (10 $\mu\text{mol/L}$), chymotrypsin (10 U/mL), apyrase (40 $\mu\text{L/mL}$), EDTA (2.5 mmol/L), chymotrypsin plus PGE_1 , or chymotrypsin plus apyrase. No

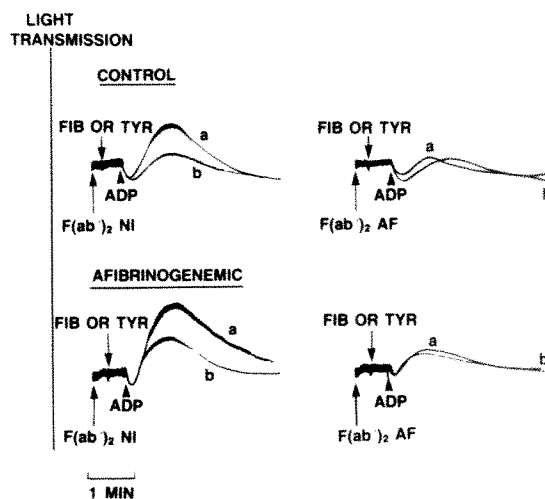


Fig 5. Platelet aggregation induced by ADP (20 $\mu\text{mol/L}$) added to suspensions of washed platelets in a medium containing 2 mmol/L Ca^{2+} , 1 mmol/L Mg^{2+} , albumin (0.35%), and apyrase. Upper pairs of tracings: Normal platelets. Lower pairs of tracings: Afibrinogenemic platelets (Patient II). (a) Represents the response of platelets to ADP in the presence of added fibrinogen (FIB, 8 $\mu\text{g/mL}$). (b) Represents the response of platelets to ADP in the absence of added fibrinogen. Tyrode's solution (TYR) was added to control samples to maintain constant volumes. The additions were made at the points indicated by the arrows. The concentration of the F(ab')_2 fragments of nonimmune IgG (F(ab')_2 NI) was 150 $\mu\text{g/mL}$; the concentration of the F(ab')_2 fragments of an antibody to fibrinogen (F(ab')_2 AF) was 100 $\mu\text{g/mL}$.

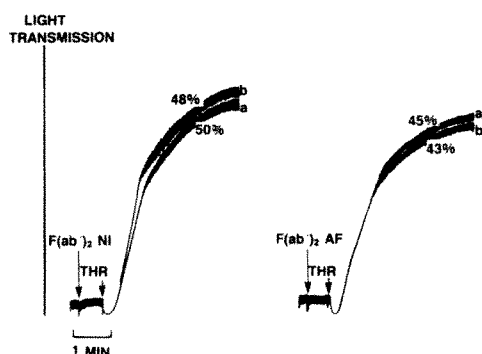


Fig 6. Platelet aggregation induced by thrombin (0.2 U/mL) added to suspensions of washed platelets in a medium containing 2 mmol/L Ca^{2+} , 1 mmol/L Mg^{2+} , albumin 0.35%, and apyrase. (a) Represents the response of platelets from a normal control. (b) Represents the response of platelets from a patient with afibrinogenemia (Patient II). The percent release of serotonin is indicated beside the aggregation tracings. The concentration of the F(ab')_2 fragments of nonimmune IgG (F(ab')_2 NI) was 150 $\mu\text{g/mL}$; the concentration of the F(ab')_2 fragments of an antibody to fibrinogen (F(ab')_2 AF) was 100 $\mu\text{g/mL}$.

combination of inhibitors was more effective in deaggregating the afibrinogenemic platelets than the control platelets.

DISCUSSION

The results from these studies show that fibrinogen in the ambient fluid is not necessary for ADP-induced platelet aggregation, although fibrinogen greatly enhances the extent of aggregation. This is in agreement with the results of Soria et al.¹

In citrated PRP from an afibrinogenemic patient and with suspensions of either normal or afibrinogenemic platelets without added fibrinogen, the formation of the small aggregates induced by ADP is not inhibited by F(ab')_2 fragments of an antibody to fibrinogen, although these fragments block the enhancement of aggregation produced by adding fibrinogen to the suspending fluid. The aggregation that occurs

without added fibrinogen is blocked by an antibody (10E5) to glycoprotein IIb/IIIa, indicating that the receptor for adhesive proteins is involved in this response. Thus there is a component of platelet aggregation that is not dependent on plasma proteins such as fibrinogen but is dependent on glycoprotein IIb/IIIa.

Washed human platelets stimulated by ADP in the presence of physiologic concentrations of Ca^{2+} release little or no serotonin from their amine storage granules and only about 4 μg of fibrinogen per 10^9 platelets from the α granules in response to 10 $\mu\text{mol/L}$ ADP.²⁸ The afibrinogenemic platelets had approximately 10 μg of fibrinogen per 10^9 platelets. If this were in the granules and released in proportion to the release from normal platelets, the concentration of fibrinogen in the ambient fluid would be less than 0.05 $\mu\text{g/mL}$. This is insufficient to cause a detectable enhancement of ADP-induced aggregation, for which at least 3 $\mu\text{g/mL}$ is necessary. Furthermore, the F(ab')_2 fragments of an antibody to fibrinogen did not inhibit the response to ADP. However, proteins other than fibrinogen, including thrombospondin, fibronectin, and von Willebrand factor (vWF) may have been released. Since fibronectin has not been found to support ADP-induced aggregation,^{45,46} it is unlikely to be involved in the aggregation observed. Recently de Marco and his associates⁴⁷ reported that addition of vWF to afibrinogenemic citrated PRP supports ADP-induced aggregation of afibrinogenemic platelets. However, the concentrations of vWF required (up to 60 $\mu\text{g/mL}$) were higher than would be present in plasma even if all the platelet vWF were released. The observation that the antibody LJP5, an antibody to glycoprotein IIb/IIIa that blocks the binding of vWF but not of fibrinogen to IIb/IIIa, inhibits platelet aggregation in response to ADP and collagen in afibrinogenemic citrated PRP is in favor of vWF being involved.⁴⁷ However, the concentration of vWF would be negligible in suspensions of washed human platelets in a medium containing 2 mmol/L Ca^{2+} , in which ADP causes very little release of α -granule contents; in addition, vWF does not support ADP-induced aggregation in this medium.⁴⁸

The response of afibrinogenemic platelets to thrombin (0.2 to 1 U/mL) or PAF (0.25 $\mu\text{mol/L}$) is practically indistinguishable from the response of normal platelets, indicating that released fibrinogen plays little part in the formation of platelet aggregates induced by these agonists. With collagen or sodium arachidonate the lack of releasable fibrinogen may contribute to the diminished aggregation response. One could also speculate that fibrinogen plays a greater part in aggregation that is largely dependent on thromboxane A_2 ; thrombin and PAF can act through pathways that are largely independent of thromboxane A_2 .⁴⁹⁻⁵¹ The finding that an antibody to fibrinogen did not inhibit platelet aggregation caused by 0.2 to 1 U/mL thrombin is in keeping with the observations of Legrand et al.³⁰ and raises the possibility, as proposed by Soria et al.,¹ of there being fibrinogen-independent mechanisms of platelet aggregation when platelets are stimulated with thrombin, and possibly with PAF.

We have previously shown that ADP-induced aggregation of human platelets is followed by rapid deaggregation if the release reaction does not occur.³⁴ Fibrinogen binding is

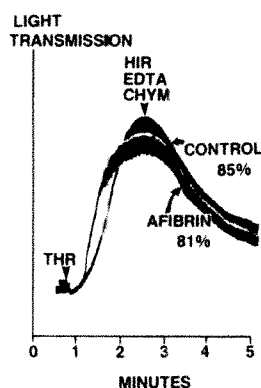


Fig 7. Effect of EDTA (2.5 mmol/L) and chymotrypsin (CHYM; 10 U/mL) on deaggregation of suspensions of washed platelets from a control subject and patient II, with afibrinogenemia. The platelets were aggregated by thrombin (1 U/mL). Hirudin (10 U/mL) was added to neutralize the thrombin two minutes after the thrombin addition, and chymotrypsin and EDTA were added with the hirudin.

associated with aggregation, and fibrinogen is lost from the surface of the platelets when they deaggregate.^{12,34} However, when the release reaction has taken place, agents such as PGE₁ or EDTA do not cause deaggregation, although they displace most of the bound fibrinogen (these agents do cause rapid reversal of ADP-induced aggregation under conditions in which the release reaction does not occur).³⁴ It was possible to study the role of fibrinogen in forming stable platelet aggregates by comparing the susceptibility of the afibrinogenemic platelets and normal platelets to deaggregation after thrombin stimulation. Since the rate at which platelets from normal and afibrinogenemic subjects deaggregated was similar, it seems reasonable to conclude that fibrinogen is not necessary for the formation of stable platelet aggregates when the platelet-release reaction has occurred. This is supported by the observation of Turitto and his associates³¹ who showed that large, stable, platelet aggregates

formed on the subendothelium of rabbit aorta exposed to native afibrinogenemic blood in a Baumgartner flow chamber.

Thus ADP-induced aggregation can occur to a small extent without the presence of fibrinogen, but it is greatly enhanced by the addition of fibrinogen. Furthermore, with agonists that cause the release reaction, enhancement by fibrinogen is seen only with low concentrations of the agonist, and fibrinogen is not required to stabilize the platelet aggregates that form under these conditions.

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HLA Associations With Leukemia

By Mortimer M. Bortin, Joseph D'Amato, Fritz H. Bach, Alfred A. Rimm, and Jon J. van Rood

Frequencies of 35 HLA A, B, C, and DR antigens were determined in 1,834 leukemic Caucasoids to evaluate possible associations between HLA and leukemia. In comparison with the frequencies of HLA antigens in published controls, the frequency of Cw3 was significantly higher in patients with acute lymphoblastic leukemia (relative risk = 2.64, $P < 0.0002$), acute myelogenous leukemia (relative risk = 1.92, $P < 0.0007$), and chronic myelogenous leukemia (relative risk = 2.07, $P < 0.002$; P values adjusted for multiple comparisons). The frequency of Cw4 was elevated in patients with acute lymphoblastic leukemia (relative risk = 2.01, $P < 0.0003$), acute myelogenous leukemia

(relative risk = 2.06, $P < 0.0002$), and chronic myelogenous leukemia (relative risk = 2.14, $P < 0.0008$). The frequency of Aw19 was significantly decreased in patients with acute myelogenous leukemia (relative risk = 0.68, $P < 0.01$) and chronic myelogenous leukemia (relative risk = 0.59, $P < 0.005$). None of the other 32 HLA antigens investigated had a statistically significant association with leukemia. The data suggest that Cw3 and Cw4 may be markers for leukemia susceptibility genes, while Aw19 may be a marker for decreased susceptibility to leukemia.

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IN 1930 Fisher¹ proposed that selective interactions between genes might favor their linkage. Darlington and Mather² extended this idea and introduced the term "supergene" for groups of linked genes that are inherited *en bloc*. Overviews of the HLA supergene were published by Ceppellini,³ Bach and van Rood,⁴ Bodmer,⁵ and Bach.⁶ The biological importance of supergenes is emphasized by the fact that they are found in all vertebrate species that have been studied. The homology between the major histocompatibility complex (MHC) in different species supports the hypothesis that the MHC has been conserved throughout phylogeny because it confers important biological advantages on the species.⁷⁻¹⁰

The observation by Lilly et al¹¹ that increased or decreased susceptibility of mice to Gross virus-induced leukemia was linked to H-2^k and H-2^b respectively prompted Amiel¹² in 1967 to search for associations between histocompatibility antigens and pathologic conditions in humans. He reported a significant association for HLA B5 in patients with Hodgkin's disease.¹² Since then numerous studies investigated HLA associations with disease.¹³⁻¹⁵

In 1984 we reported the results of analyses investigating HLA associations with leukemia in 1,009 leukemia patients¹⁶ (hereafter referred to as "initial series"). We found a statistically significant increase in the frequency of Cw3 and Cw4 in patients with acute lymphoblastic leukemia (ALL) and of Cw4 in patients with acute myelogenous leukemia (AML). An additional 825 leukemia patients have now been studied ("current series"), and the results from these patients and the combined series are presented here.

PATIENTS AND METHODS

Patients. Detailed information regarding 1,834 North American and European Caucasoid patients treated with chemoradiotherapy and bone marrow transplantation (BMT) for leukemia was reported to the International Bone Marrow Transplant Registry. Transplants were performed between 1969 and 1985 by 96 BMT teams worldwide. The diseases studied were ALL, AML, and chronic myelogenous leukemia (CML; Table 1). Patients' median age was 21.4 years (range 0.6 to 59 years), and 55% were males. The median interval from diagnosis to transplantation was 9 months (range 0.3 to 144 months). At the time of transplantation 47% of the patients were in first remission or chronic phase, 20% in second remission, and the disease was more advanced in 33%. Chemotherapy failed to induce a first remission in 5% of the patients with acute leukemia.

Antigenic splits were converted to their equivalent broad specificities because many of the splits were not defined in the earlier years of this study. Data for 35 HLA antigens were available for investigation: eight HLA A antigens (1,2,3,9,10,11,w19,28), 15 HLA B antigens (5,7,8,12,13,14,15,16,17,18,21,w22,27,35,40), six HLA C antigens (w1,w2,w3,w4,w5,w6), and six HLA DR antigens (1,2,3,4,5,7). Estimates of the frequency of DRw6 in North American and European Caucasoid controls were not thought to be reliable, and this antigen was excluded from analysis. Data were available to analyze the frequency of HLA A in 1,834 patients, B in 1,801 patients, C in 783 patients, and DR in 709 patients.

HLA antigen frequencies for the control series of North American and European Caucasoids were obtained from Tables 6 through 9 in Baur and Danilovs¹⁷ population analysis from the Eighth International Histocompatibility Workshop.

Statistical analysis. The extensive polymorphism of HLA not only provides a large number of markers to be tested for possible associations with diseases but also presents problems in interpretation of results. The main problem is that the performance of large numbers of statistical comparisons increases the probability that apparent differences between two groups may have occurred solely by chance. Svejgaard et al¹⁸ discussed this problem and suggested several methods to help resolve it.

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Table 1. Distribution of Patients by Disease

Disease	Initial Series	Current Series	Total
ALL	423	304	727
AML	383	282	665
CML	203	239	442
Total	1,009	825	1,834

Haldane's modification¹⁹ of Woolf's method²⁰ was used to assess the significance of the differences in the antigen distributions in the control v the leukemia populations. Multiplex testing for heterogeneity in a single locus or multiple loci was performed by summing the chi squares provided by the Woolf-Haldane analysis. That value is itself a chi square with degrees of freedom equal to the total number of antigens in the locus or loci.²¹ Corrected *P* values were derived by taking into account the 35 antigens that were tested using the method of Edwards.²² Only corrected *P* values are presented.

Initially HLA antigen frequencies from the 258 North American and the 567 European Caucasoids from the current series were tested independently against their respective controls. Comparisons were then made with the HLA antigen frequencies found in the 628 North American and 381 European Caucasoids from the initial series. This strategy was used because it would be most unlikely that the same association would be observed by chance alone in all four subsets. All four subsets were combined for an overall analysis.

RESULTS

Multiplex testing disclosed no significant heterogeneity in the distribution of HLA A, B, C, and DR antigens between the North American and European patients in the initial or current series. In particular there were no significant differences for the Aw19, Cw3, and Cw4 antigens. The distribution of the Aw19, Cw3, and Cw4 antigens in the North American and European control populations also did not differ significantly.

Of the 35 HLA antigens studied, only Aw19, Cw3, and Cw4 had significant associations with leukemia in general, ie, the combined diseases ALL, AML, and CML (Table 2). Compared with controls, Aw19 had a lower frequency and decreased relative risk in all four subsets of patients; the pooled data had a frequency of 23% v 30% for controls with a relative risk of 0.71 and a *P* value < 0.0006. Both Cw3 and Cw4 had consistently elevated frequencies in all four subsets in comparison with controls, and the relative risks of 2.20 and 2.06 respectively for the pooled data were highly significant (*P* < 0.00001).

Shown in Table 3 are the relative risks and significance levels for Aw19, Cw3, and Cw4 in the initial, current, and

combined series for ALL, AML, and CML. For the Aw19 antigen, note that the relative risk was consistently below 1.0 in each of the North American and European subsets and for each of the diseases studied, but in none of the 12 subsets was statistical significance observed. It was not until the data were pooled, with a resultant increase in the power of the test, that statistically significant lower frequencies for Aw19 were found both for the 665 patients with AML and the 442 patients with CML. In the pooled data from 727 patients with ALL, the frequency of Aw19 was lower than controls, but the level was not statistically significant.

The frequency for Cw3 was consistently elevated for patients with ALL, AML, and CML but was statistically significant in only three of four ALL, in one of four AML, and in one of four CML subsets (Table 3). When the data were pooled, the power of the test increased, and the frequency of Cw3 was significantly increased for each of the diseases. Similarly, the frequency of Cw4 was consistently elevated for all 12 subsets of patients but was statistically significant in only two subsets of AML patients. The combined data indicate that Cw4 has a significantly increased frequency for ALL, AML, and CML.

In the combined series there were 72 patients with ALL or AML in whom complete remission was not attained following induction chemotherapy. They were considered to be refractory to chemotherapy. To determine whether Aw19, Cw3, or Cw4 might be associated with responsiveness to chemotherapy in leukemia patients rather than leukemia per se, the frequencies of these antigens were analyzed in the 1,320 patients with acute leukemia who had responded to chemotherapy and the 72 patients who had not. No statistically significant heterogeneity was found; chi squares were 1.08 for Aw19; 0.03 for Cw3; and 0.01 for Cw4. Among the patients who had not responded to chemotherapy, the frequency of Aw19 was not significantly different from patients who had responded; 31% v 30% respectively. The frequency of Cw3 of nonresponders, however, was increased; 36% v 20% for controls (uncorrected *P* = 0.012), as was the frequency of Cw4; 40% v 22% for controls (uncorrected *P* = 0.005).

DISCUSSION

This investigation of 1,834 patients with leukemia disclosed highly significant associations between HLA and the risk of leukemia. In comparison with healthy controls, frequencies of Cw3 and Cw4 were increased (relative risk > 2.0, *P* < 0.00001), and the frequency of Aw19 was decreased

Table 2. Woolf-Haldane Analysis of HLA Antigens Significantly Associated with ALL, AML, and CML

Antigen	C (%) [*]	Initial Series				Current Series				Combined Series		
		North American		European		North American		European		L (%)	RR	<i>P</i> [§]
		L (%) [†]	RR [‡]	L (%)	RR	L (%)	RR	L (%)	RR			
Aw19	30	22	0.68	24	0.75	24	0.76	23	0.71	23	0.71	<.0006
Cw3	20	37	2.01	34	2.18	34	1.80	36	2.40	36	2.20	<.00001
Cw4	22	35	2.06	41	2.35	39	2.41	35	1.86	37	2.06	<.00001

^{*}Antigen frequencies for North American and European controls.¹⁷

[†]Antigen frequencies for patients with leukemia.

[‡]Relative risk.

[§]Corrected for 35 comparisons.

Table 3. Woolf-Haldane Analysis of Aw19, Cw3, and Cw4 Associations with Leukemia

Antigen	Disease	Initial Series				Current Series				Combined Series	
		N American		European		N American		European		RR	P
		RR*	P†	RR	P	RR	P	RR	P		
Aw19	ALL	0.84	NS	0.88	NS	0.64	NS	0.93	NS	0.84	NS
Aw19	AML	0.62	NS	0.67	NS	0.91	NS	0.64	NS	0.68	<.01
Aw19	CML	0.49	NS	0.68	NS	0.83	NS	0.55	NS	0.59	<.005
Cw3	ALL	2.54	.002	3.19	.002	1.33	NS	2.84	.003	2.64	<.0002
Cw3	AML	1.34	NS	2.00	NS	2.22	NS	2.25	.03	1.92	<.0007
Cw3	CML	3.44	.01	1.14	NS	2.37	NS	1.78	NS	2.07	<.002
Cw4	ALL	2.01	NS	2.26	NS	2.58	NS	1.82	NS	2.01	<.0003
Cw4	AML	2.46	.006	2.52	.018	1.78	NS	1.72	NS	2.06	<.0002
Cw4	CML	1.41	NS	2.29	NS	3.59	NS	2.08	NS	2.14	<.0008

*Relative risk.

†Corrected for 35 comparisons.

(relative risk <0.72, $P < 0.0006$). These findings confirm and extend our previous report investigating HLA association with leukemia.¹⁶ Frequencies for the 32 other A, B, C, and DR antigens studied were not significantly different from controls.

Most studies of HLA associations with leukemia have focused on the HLA A, B, or D/DR loci.²³⁻⁴¹ The findings have been inconsistent, and no strong associations between these loci and leukemia have been reported (Table 4). In particular, to the best of our knowledge, associations between HLA C and leukemia were not reported prior to or since our initial study.¹⁶ De Jongh et al²⁶ conducted the only study known to us in which HLA C associations with leukemia were investigated. They found no significant difference in HLA C antigen frequency between controls and a group of 44 children with ALL. The discrepancy between their results and ours may have been due to the low power of the test in their study.

The pooled data showed that Aw19 had a significantly lower frequency among leukemia patients in general. In our initial study the lower frequency of Aw19 in leukemia patients was uncertain because it had only borderline significance (corrected $P = 0.047$) in the North American subset of 628 patients and was not significant in the 381 European patients.¹⁶ With the group size increased from 1,009 to 1,834 leukemia patients, it now seems clear that the frequency of Aw19 is indeed decreased in leukemia patients. The frequency of Aw19 was significantly reduced in AML ($P < 0.01$) and CML ($P < 0.005$). Although not statistically significant in ALL, it should be noted that the relative risk associated with Aw19 was consistently lower than 1.0 in all four subsets of ALL patients (Table 3). In one study of 60 patients with ALL, a decreased frequency of Aw19 (uncorrected $P < 0.05$) was reported by Klouda et al.³⁵ It is possible that when additional patients with ALL become available for study, Aw19 may also prove to be associated with a reduced risk for this disease.

The strong positive association of Cw3 and Cw4 with leukemia supports the possibility that these antigens, or the genes encoding them, are, or are closely linked with, possible susceptibility genes. An increased relative risk of leukemia has been observed in the siblings of leukemia patients,^{42,43} but the mechanism is not known. Based on the findings reported

here, it is possible that the mechanism may be due in part to an increased frequency of Cw3 or Cw4 in siblings of leukemia patients. Studies of HLA C in instances of familial leukemia are encouraged. Another possibility, suggested by other studies, is that Aw19, Cw3, and Cw4 may be markers coding for immune responsiveness to putative leukemia viruses; DeVries et al⁴⁴ found a significant association between Cw3 and low in vitro responses to vaccinia virus. Our data show no association between Aw19, Cw3, or Cw4 and responsiveness to chemotherapy in leukemia patients.

The reader is cautioned that our findings may not be representative of all leukemia patients because of several unavoidable biases in the database of the International Bone Marrow Transplant Registry. For example, most teams do not perform BMTs on older patients. As a consequence, almost all patients in this study were less than 50 years of age, and our findings may not apply to older leukemia patients. The 95% remission-induction rate in our cases with acute leukemia is higher than most reports of nontransplanted patients, especially those with AML. All patients reported here survived long enough to undergo BMT. It is likely that some patients for whom BMT was planned died before the plan could be implemented. It is possible that patients with especially virulent forms of leukemia or, as suggested by Rogentine et al,³³ some less hardy patients did not survive long enough to be transplanted and thus were not available to us for study. Other interpretations are possible. Furthermore, HLA data for patients with chronic lymphocytic leukemia were not available to us for analysis, and it is not known whether the findings reported here also apply to these patients or to non-Caucasoids. Studies of HLA associations with morphological, immunologic, and cytogenetic subtypes of leukemia will be undertaken in the future with an expanded database.

The positive and negative associations with leukemia we found with relative risks ranging from 2.64 to 0.59, while highly significant, are weak in comparison with those described in some other studies showing significant associations between HLA and disease.¹³⁻¹⁵ The extremes, according to Svejgaard et al,¹⁴ are the positive association between B27 and ankylosing spondylitis, with a relative risk of 87.4, and the negative association between DR2 and insulin-dependent diabetes, with a relative risk of 0.2. The analyses presented

Table 4. Summary of Representative Reports in Which Associations Between HLA and Leukemia Were Investigated

Disease	No. Patients	No. Antigens Studied	Significant Antigen	Frequency*	P	Corrected†	Ref
ALL	28	27 A, B	None	—	—	—	23
ALL	74	13 DR	None	—	—	—	24
ALL	28	34 A, B	None	—	—	—	25
ALL	44	65 A, B, C, DR	None	—	—	—	26
ALL	102	10 A, B	None	—	—	—	27
ALL	58	18 A, B	None	—	—	—	28
ALL	94	25 A, B	None	—	—	—	29
ALL	20	32 A, B, DR	A1	Increased	<.02	No	30
ALL	42	20 A, B	A2	Increased	<.09	No	31
ALL	198	25 A, B	A2	Increased	<.02	No	32
ALL	85	20 A, B	A2	Increased	<.005	Yes	33
ALL	98	14 A, B	A2	Increased	<.001	No	34
ALL	42	20 A, B	A3	Increased	<.09	No	31
ALL	198	25 A, B	A9	Increased	<.03	No	32
ALL	98	14 A, B	A9	Decreased	<.01	No	34
ALL	60	21 A, B	A9	Decreased	<.04	No	35
ALL	34	22 A, B	A11	Decreased	<.02	No	36
ALL	60	21 A, B	Aw19	Decreased	<.05	No	35
ALL	60	21 A, B	B8	Increased	<.04	No	35
ALL	98	14 A, B	B8	Increased	<.005	No	34
ALL	98	14 A, B	B12	Increased	<.001	No	34
ALL	31	32 A, B, DR	B13	Decreased	<.05	No	30
ALL	727	35 A, B, C, DR	Cw3	Increased	<.0002	Yes	‡
ALL	727	35 A, B, C, DR	Cw4	Increased	<.0003	Yes	‡
AML	150	25 A, B	None	—	—	—	37
AML	179	25 A, B	None	—	—	—	32
AML	136	28 A, B	None	—	—	—	38
AML	33	22 A, B	A2	Increased	<.01	No	36
AML	33	22 A, B	A11	Decreased	<.05	No	36
AML	26	20 A, B	B14	Increased	<.05	Yes	31
AML	38	32 A, B, DR	B21	Increased	<.05	No	30
AML	38	32 A, B, DR	Bw22	Increased	<.01	No	30
AML	38	32 A, B, DR	DR3	Increased	<.05	No	39
AML	665	35 A, B, C, DR	Aw19	Decreased	<.01	Yes	‡
AML	665	35 A, B, C, DR	Cw3	Increased	<.0007	Yes	‡
AML	665	35 A, B, C, DR	Cw4	Increased	<.0002	Yes	‡
CML	60	20 A, B	None	—	—	—	31
CML	15	22 A, B	A1	Increased	<.02	No	36
CML	47	26 A, B	A3	Increased	<.05	No	40
CML	47	26 A, B	B12	Decreased	<.01	No	40
CML	442	35 A, B, C, DR	Aw19	Decreased	<.005	Yes	‡
CML	442	35 A, B, C, DR	Cw3	Increased	<.002	Yes	‡
CML	442	35 A, B, C, DR	Cw4	Increased	<.0008	Yes	‡
ALL/AML	89	32 A, B, DR	B15	Decreased	<.05	No	30
ALL/AML	89	32 A, B, DR	B21	Increased	<.05	No	30
ALL/AML	89	32 A, B, DR	DR5	Decreased	<.05	No	30
ALL/AML	114	8 D	Dw7	Increased	<.05	No	41
ALL/AML/CML	1,834	35 A, B, C, DR	Aw19	Decreased	<.0006	Yes	‡
ALL/AML/CML	1,834	35 A, B, C, DR	Cw3	Increased	<.00001	Yes	‡
ALL/AML/CML	1,834	35 A, B, C, DR	Cw4	Increased	<.00001	Yes	‡

*Compared with healthy controls.

†P value corrected for multiple comparisons.

‡This report.

here clearly show the desirability of a large database in studies investigating HLA associations with disease when there is in fact a relationship but it is not strong. For example, in Table 3, although a trend was present, Aw19 was not significantly associated with AML or CML in any of the subsets. Only when group sizes in the combined series were

665 (AML) and 442 (CML) did statistically significant associations become evident. Similarly, Cw4 was not statistically significant in any of the ALL or CML subsets; using pooled data from the combined series demonstrated highly significant associations with each disease. Another problem with relatively small group sizes is demonstrated by those

instances when significant associations were found in some subsets but not in others (Table 3). It is clear from the data shown in Table 4 that inadequate group sizes account for many of the conflicting reports in the literature describing the presence or absence of HLA associations with leukemia.

The increased frequency of Cw3 and Cw4 and decreased frequency of Aw19 in leukemia patients provides strong evidence that the HLA supergene, as H-2 in the mouse, may play a role in increased and decreased susceptibility to leukemia. Although it is far from clear that the mechanism for the association in mouse and man is similar, until now the parallel has been elusive.

It is generally believed that HLA antigens influence the T lymphocyte repertoire. We found that the risk of leukemia is significantly increased in individuals with Cw3 or Cw4. It seems likely that these people will have a T cell repertoire that is different from individuals who are negative for Cw3 and Cw4. This simply means that Cw3 and Cw4 antigens are immune response gene products. It does not answer the question of how these immune response genes confer low responsiveness against a putative leukemia-inducing agent. Our data imply that individuals who have neither Cw3 nor Cw4 are better able to resist leukemia than those who are positive. This remains purely speculative because there are no available tests to investigate this hypothesis.

The fundamental importance of this study is not that Cw3 and Cw4 were found to have significant associations with leukemia but rather that HLA C genes, or genes closely linked with HLA C, have hitherto unrecognized properties. Whether HLA C also will be found to be associated with other malignant and nonmalignant diseases remains to be determined.

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APPENDIX

This 32nd report from the International Bone Marrow Transplant Registry was prepared for the Advisory Committee: R.P. Gale, Los Angeles (Chairman); F.H. Bach, Minneapolis; A.J. Barrett, London; D.W. van Bekkum, Rijswijk; J.C. Biggs, Sydney; K.G. Blume, Los Angeles; M.M. Bortin, Milwaukee; K.A. Dicke, Houston; E. Gluckman, Paris; J.M. Goldman, London; R.A. Good, St. Petersburg; R. Hong, Madison; R.H. Herzig, Cleveland; J.H. Kersey, Minneapolis; H.J. Kolb, Munich; A.M. Marmont, Genoa; T. Masaoka, Osaka; H.A. Messner, Toronto; R.J. O'Reilly, New York; R.L. Powles, London; A.A. Rimm, Milwaukee; O. Ringden, Huddinge; J.J. van Rood, C. Rozman, Barcelona; B. Speck, Basel; R.S. Weiner, Gainesville; and F.E. Zwaan, Leiden.

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Identification of Molecular Variants of p210^{bcr-abl} in Chronic Myelogenous Leukemia

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The aberrant *abl* protein product of a chronic myelogenous leukemia (CML) blast crisis cell line (K562) and of five Philadelphia chromosome-positive CML patients in blast crisis were analyzed by an immune complex kinase assay using two antipeptide sera generated against the hydrophilic domain of *v-abl* and a region within the third exon of the breakpoint cluster region (*bcr*) respectively. Both the anti-*abl* and anti-*bcr* sera detected a 210 kd band in extracts derived from K562 cells and from two CML patients with myeloid blast crisis. p210 was detected by the anti-*abl* but not the anti-*bcr* sera in three CML patients

A SHORTENED chromosome 22, designated the Philadelphia (Ph) chromosome, is found in the majority of patients with chronic myelogenous leukemia (CML) and is considered the cytogenetic hallmark of the disease.^{1,2} This chromosomal anomaly is usually the result of a reciprocal translocation between chromosomes 9 and 22, (t[9;22](q34;q11)).² The breakpoints on chromosome 22 occur within a 5.8 kb segment of DNA, termed the breakpoint cluster region or *bcr*.^{3,4} A crucial event in the Ph translocation is the transfer of the *c-abl* oncogene from its normal residence on chromosome 9 to chromosome 22 so that *bcr* and *c-abl* sequences are juxtaposed in a head-to-tail configuration with *bcr* closer to the centromere.^{5,6} The resulting messenger RNA (mRNA) is a hybrid of 5' *bcr* sequences and a truncated *c-abl* gene lacking the first exon.⁶ This chimeric *bcr-abl* mRNA encodes a novel 210,000 MW protein, whereas the normal *c-abl* gene is expressed as a 145,000 MW protein.⁷⁻⁹ Therefore p210^{bcr-abl} represents a unique cancer-specific marker.

The Abelson murine leukemia virus encoded p160^{v-abl} has tyrosine-specific protein kinase activity that is closely associated with its ability to transform cells.¹⁰⁻¹³ Although both the aberrant p210^{bcr-abl} and the normal p145^{c-abl} also have an associated tyrosine-protein-kinase activity, p160^{v-abl} and p210^{bcr-abl} utilize themselves as substrates differently than p145^{c-abl} in vitro.⁹ The high specificity and altered autophosphorylation properties of p210^{bcr-abl} strongly implicate a critical role for this molecule in the pathogenesis of CML.

In this report we have used anti-*abl* and anti-*bcr* (to exon 3) peptide sera in an immune complex kinase assay (ICKA) to investigate p210^{bcr-abl} expression in Ph-positive CML. We demonstrate that a variable number of *bcr* exons may be incorporated into p210^{bcr-abl} and that the resulting heterogeneous products retain enzymatic activity as protein kinases.

MATERIALS AND METHODS

Antisera. Antiserum was made against a peptide representing the predicted hydrophilic *v-abl* sequence 389-403 (DEVEKELGKRGTRGG-C) and a region within *bcr* exon 3 (DDESPGLYGLNV-C),⁶ as described by Kloetzer et al.⁷ The peptide was synthesized using solid-phase synthesis. Cysteine residues were added to the carboxyl termini during synthesis for MBS cross-linking through free sulfhydryl groups to the carrier protein keyhole limpet hemocyanin. Antigen was prepared by emulsifying at 1:1

with myeloid (one patient) and lymphoid (two patients) blast crisis, indicating the absence of *bcr* exon 3 in this protein. Southern blot analysis on DNA derived from one of the patients in the latter group was consistent with the break on chromosome 22 occurring 5' to *bcr* exon 3. Our observations demonstrate that the Philadelphia translocation results in the generation of a chimeric *bcr-abl* protein with at least two molecular variants, both of which are enzymatically active as protein kinases.

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ratios 0.2 mg of cross-linked peptide in Dulbecco's phosphate-buffered saline (PBS) with 1.5 mL of complete Freund's adjuvant. Subcutaneous (SC) injections of rabbits were administered at multiple sites. Three booster injections of antigen suspended in incomplete Freund's adjuvant were given at 2-week intervals.

Immune complex kinase assays (ICKAs). Blood samples were obtained from patients after informed consent. The blood was prepared for ICKAs by treating with 5 volumes of 1.22% ammonium oxalate to lyse red blood cells (RBCs) and Ficoll-Hypaque (FH) gradient centrifugation to enrich for immature cells. K562 cells (Ph-positive CML erythroid blast crisis cell line)^{14,15} were also assayed. Each sample was divided into four aliquots of 5×10^7 cells. Each aliquot was disrupted with 1 mL of lysis buffer (1% Triton X-100, 5 mmol/L EDTA, 100 mmol/L NaCl, 5 mmol/L phenylmethylsulfonylfluoride, 100 KIU/mL Trasylol, in 10 mmol/L sodium phosphate, pH 7.5) with 20 strokes in a tight-fitting Wheaton homogenizer. Lysates were subjected to a 10,000 g centrifugation for ten minutes at 4°C. The supernatant from the four aliquots was then incubated with (1) 5 μ L of anti-*abl*389-403 serum, (2) 5 μ L of anti-*abl*389-403 serum and 5 μ L of *abl*389-403 peptide (3) 5 μ L of anti-*bcr*3 serum, or (4) 5 μ L of anti-*bcr*3 serum and 5 μ L of *bcr*3 peptide. Concentration of the peptide solution was 1 mg/mL H₂O. Incubation was carried out for one hour on ice. The resulting immune complexes were then precipitated by incubation with 10 μ L of Pansorbin for 20 minutes on ice. Immunoprecipitates were washed twice with wash buffer (0.1% Triton X-100, 150 mmol/L NaCl, in 10 mmol/L sodium phosphate, pH 7.5). The pellets were drained, resuspended in 50 μ L of 20 mmol/L HEPES (pH 7.0), containing 0.1% Triton X-100, and 150 mmol/L NaCl, and then reacted with

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50 μ L of a mixture containing 5 μ Ci [gamma- ^{32} P] ATP (50 Ci/mmol), 20 mmol/L $MnCl_2$, 0.1% Triton X-100 in 20 mmol/L HEPES (pH 7.0) for ten minutes on ice. The reaction was terminated by addition of 3 mL of buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% DOC, 100 mmol/L NaCl, 5 mmol/L EDTA, in 10 mmol/L sodium phosphate, pH 7.5. The immune complexes were washed twice with the above buffer and then prepared for electrophoresis according to Laemmli.¹⁶ Phosphoproteins were electrophoretically resolved according to mol wt on an 8% SDS polyacrylamide gel and sized with prestained mol wt markers. The gel was exposed overnight on Kodak XAR-5 film.

Southern blotting. DNA was prepared by cell lysis, proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation as previously described.¹⁷ Twenty micrograms of genomic DNA was digested with BglIII and Bam HI restriction endonucleases (2 U/ μ g of DNA) in conditions recommended by the supplier (International Biotechnologies, Inc, New Haven, CT), size fractionated by electrophoresis in a 0.8% agarose gel, denatured and transferred to nitrocellulose as described by Southern.¹⁸ A 3', 1.2kb *bcr* (HindIII-BglIII) plasmid probe (Oncogene Science, Inc, Mineola, NY) was used.³ The probe was nick-translated to a specific activity of 1 to 2×10^8 cpm/ μ g of DNA. Hybridization was carried out overnight at 42°C in the presence of 10% dextran sulfate. The filters were then washed twice at 60°C for 30 min/wash in $0.1 \times$ standard saline citrate (SSC) ($1 \times$ SSC = 0.15 mol/L sodium chloride/0.015 mol/L sodium citrate, pH 7) containing 0.1% SDS, air dried, and autoradiographed.

RESULTS

A 210,000 MW *abl* protein was detected in extracts of K562 cells using anti-*abl*389-403 serum in an ICKA (Fig 1, lane 1), a result consistent with previous reports.⁷⁻⁹ The 190,000 MW protein seen on the gels is thought to be either a proteolytic fragment of p210 or a second translation product of the *bcr-abl* gene. The 210,000 and 190,000 MW proteins were determined to be *abl* specific, since these proteins were not detected in assays in which the reactivity of the anti-*abl*389-403 serum was blocked by prior addition of excess *abl*389-403 peptide (Fig 1, lane 2). Addition of an unrelated peptide did not change the reactivity of anti-*abl*389-403 serum with p210 or p190 (Fig 1, lane 5), demonstrating that the blocking observed after addition of cognate peptide is not due to nonspecific competition. Using identical assay conditions, p210 was also detected in K562 cell extracts when the anti-*bcr*3 serum was used (Fig 1, lane 3), indicating that

p210 consists of both *bcr* and *c-abl* sequences. The incorporation of *bcr* exon 3 in K562 p210^{*bcr-abl*} is supported by the detailed investigations of Shtivelman et al,⁶ indicating that the RNA junction point in the *bcr-abl* transcript derived from K562 cells includes the third *bcr* exon.

Fresh cells from five CML patients, designated A, B, C, D, and E, were available for ICKA. Patients A, D, and E had myeloid blast crisis; patients B and C, lymphoid blast crisis. Examination of cell extracts derived from patients A and E revealed results that were similar to those found in K562 cells: p210 was expressed as an enzymatically active protein kinase and could be detected by both anti-*abl*389-403 and anti-*bcr*3 sera (Fig 1, lanes 15-22). In contrast, anti-*bcr*3 serum was not able to detect p210^{*bcr-abl*} protein kinase activity in cell extracts derived from CML patients B, C, and D (Fig 1, lanes 8 and 9, 13 and 14, 25 and 26), even though this protein is expressed and enzymatically active as a protein kinase, as demonstrated by its detection by anti-*abl*389-403 in the ICKA (Fig 1, lanes 6 and 7, 11 and 12, 23 and 24). The faint 210 kd band detected in CML patient B by the anti-*bcr*3 serum (Fig 1, lane 8) represents nonspecific background phosphorylation, as demonstrated by the failure of this band to be blocked by cognate peptide (Fig 1, lane 9) and by the detection of a similar faint band by preimmune rabbit serum (Fig 1, lane 10). Our results demonstrate the existence of two molecular variants of p210^{*bcr-abl*}—*bcr*(3):*abl* and *bcr*(-3):*abl*—in different CML patients.

Southern blot analysis revealed rearrangement in *bcr* in both BglIII and BamHI digested DNA from patient A (Fig 2, lane 3). These data are consistent with the inclusion of exon 3 of *bcr* in the expressed p210^{*bcr-abl*} of this patient (Fig 3). Rearrangement in *bcr* was observed in BglIII but not BamHI digested DNA from patient B (Fig 2, lane 1). Since the BamHI restriction site is located proximal to exon 3 of *bcr* (Fig 3), the break on chromosome 22 in patient B must occur 5' to exon 3. Therefore the results obtained from Southern blot analysis are consistent with the absence of exon 3 of *bcr* in the p210^{*bcr-abl*} derived from patient B, as demonstrated by ICKA. Differences in the intensity of bands from lane to lane were similar when the filters shown in Fig 2 were reprobed with a beta-actin cDNA probe (data not shown), indicating that these differences reflected variations in the amount of DNA in each lane.



Fig 1. Immune complex kinase assays of CML cells. Lanes 1,6,11,15,19,23, anti-*abl*389-403 serum; lanes 2,7,12,16,20,24, anti-*abl*389-403 serum and *abl*389-403 peptide block; lanes 3,8,13,17,21,25, anti-*bcr*3 serum; lanes 4,9,14,18,22,26, anti-*bcr*3 serum and *bcr*3 peptide block; lane 10, preimmune rabbit serum; lane 5, anti-*abl*389-403 serum and unrelated (*bcr*3) peptide block; Cells were used as follows: lanes 1-5, K562; lanes 6-10, CML patient B; lanes 11-14, CML patient C; lanes 15-18, CML patient A; lanes 19-22, CML patient E; lanes 23-26, CML patient D.

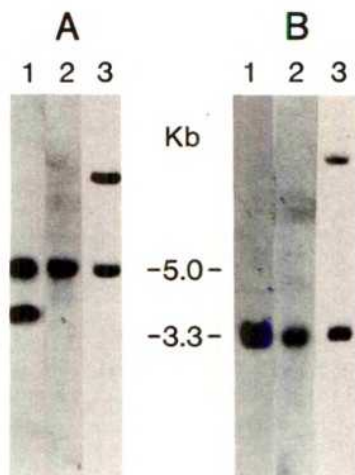


Fig 2. Southern blot analysis of DNA samples using a 3' 1.2 kb HindIII-BglII *bcr* probe. Restriction enzymes used were BglII (panel A) and Bam HI (panel B). Lane 1, CML patient B; lane 2, normal volunteer; lane 3, CML patient A.

DISCUSSION

The N-terminal replacement of *c-abl* by *bcr* sequences in the 210,000 MW protein product of Ph-positive CML patients has been previously predicted by studies of the aberrant 8 kb *bcr-abl* mRNA found in these patients.^{6,19} In addition, Ben-Neriah et al²⁰ have recently demonstrated that p210 contains both *bcr* and *c-abl* sequences. Our data indicate that the Ph translocation results in the generation of at least two molecular variants of the *bcr-abl* protein. These variants differ in that they either contain or lack *bcr* exon 3—*bcr*(3):*abl* v *bcr*(-3):*abl*. They are generated as a result of the differing locations of the breaks on chromosome 22, which can occur both proximal and distal to the third exon of *bcr*. The absence of *bcr* exon 3 does not appreciably alter the size of the 210-kd *bcr-abl* protein, probably because this exon is small, consisting of only 25 amino acids.⁴ Our experiments also demonstrate that both *bcr-abl* variants are enzymatically active as phosphokinases, a property of potential fundamental importance for CML, since this enzymatic activity has been strongly implicated in the ability of several oncogenes and growth factor receptors to exert their effects.²¹

The clinical course of CML is characterized by the inevitable progression from an easily controlled benign phase to a terminal blast transformation phase²² in a time period

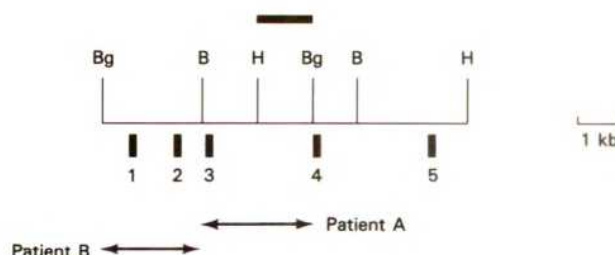


Fig 3. Restriction enzyme map of *bcr* (adapted from Heisterkamp et al⁴). Exons are indicated by vertical boxes below the map. The horizontal bar above the map shows the 1.2 kb HindIII-BglII *bcr* probe. Below the map the approximate positions of the breakpoints of Ph-positive CML patients A and B. B, Bam HI; Bg, BglII; H, HindIII.

ranging from several weeks to 23 years (median interval = 3.5 years).^{22,23} The blasts at the time of transformation usually display characteristics of myeloid lineage, although a minority of patients (about 30%) may develop a lymphoid blast crisis.²⁴ The factors influencing the morphology of the blasts and the length of time to the transformation stage remain unknown. Recent work has demonstrated that subtle alterations in oncogene configuration can be associated with phenotypic variations of a tumor, ie, distinct chromosomal 8 breakpoint sites relative to the *c-myc* oncogene are found in the endemic v sporadic forms of Burkitt's lymphoma.²⁵ Interestingly, two of three patients with the *bcr*(-3):*abl* protein variant demonstrated several unusual clinical features: a survival of 10 and 11 years respectively (seen in less than 5% of patients)²⁶ and a terminal lymphoid blast phase. Both patients with the *bcr*(3):*abl* protein showed a more typical clinical course, with a survival of 3 and 3.5 years respectively and a terminal blast crisis of myeloid lineage. Whether the absence of *bcr* exon 3 in the p210^{bcr-abl} product of some CML patients is related to long survival, and/or the development of a blast crisis of lymphoid lineage, or is merely fortuitous requires further investigation. Studies correlating the exact location of the breakpoint in *bcr* and the structure of p210^{bcr-abl} with clinical characteristics of a large series of CML patients are in progress and should help elucidate whether or not the generation of molecular variants of p210^{bcr-abl} is important in the malignant evolution of CML.

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Pseudosickling of Hemoglobin Setif

By Samuel Charache, Eva Raik, David Holtzclaw, Patricia J. Hathaway, Elizabeth Powell, and Patricia Fleming

Hemoglobin Setif produces pseudosickling of red cells in vitro; the nature of the process and the conditions that "trigger" it are unknown. Studies of red cells, hemolysates, purified hemoglobin solutions, and artificial mixtures of Hb A and Setif suggest that pseudosickling is produced by intracellular crystallization of insoluble hemoglobin. Increased tonicity of the suspending medium accentuates the process, probably by causing a rise in intracellular

hemoglobin concentration. If precipitates from A/Setif mixtures are analyzed, they always contain Hb A, suggesting an unusual mechanism for the process. Despite the fact that osmolality in the renal medulla is similar to that which produces pseudosickling in vitro, carriers do not have renal dysfunction of the type found in patients with sickle cell disease.

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PSEUDOSICKLING of red cells containing Hb Setif ($\alpha 94 \text{ Asp} \rightarrow \text{Tyr}$) was first described by Drupt et al^{1,2} and has been reported by others.³ The appearance of deformed cells does not resemble either truly sickled cells or the sickled cells of deer and other species: their contorted, elongated form gives an impression of twisted rods rather than the anisotropic domains characteristic of classic sickling disorders. Like the red cells of deer and unlike true sickle cells, they have not been found in vivo, and they have not been recognized to produce a clinical disorder.

Conditions required for deformation of Hb Setif-containing red cells have not been defined. Drupt found that incubation of a small volume of blood (50 μL) in an empty 5-mL plastic tube at 37°C yielded optimal results,⁴ and he and his coworkers showed that the phenomenon requires oxyhemoglobin, not deoxyhemoglobin.² They suggested that dehydration of red cells might be involved in sickling, and Raik et al³ showed that sickling could not be related to variations in the salt composition of isotonic suspending solutions. The requirement for oxyhemoglobin may perhaps be explained by the site of the amino acid substitution⁵: replacement of Asp $\alpha 94$ (G1) by tyrosine would be expected to disrupt a hydrogen bond that stabilizes the oxy, but not the deoxy, conformation of the hemoglobin molecule.⁶

If we are to truly understand the pathogenesis of sickle cell disease, the absence of clinical illness and of in vivo sickling in almost all persons with sickle cell trait is as important as their presence in patients with sickle cell anemia. Normal β^A globin chains, either in hybrid molecules ($\alpha_2\beta^A\beta^S$) or in homotetramers ($\alpha_2\beta_2^A$) can copolymerize with Hb S,⁷⁻¹⁰ and AS erythrocytes can sickle, but they usually do not at normal blood PO_2 , and most heterozygotes remain symptom free. Heterozygous carriers of Hb Setif are also symptom free¹¹⁻¹³; their freedom from more than minor hematologic abnormalities (see below) is usually attributed to the low proportion of the abnormal hemoglobin in their red cells (12% to 24%). Raik et al studied a compound heterozygote (II-1)³ with Setif/alpha and beta thalassemia, whose red cells contained 30% of the abnormal hemoglobin; he too showed neither vaso-occlusive crises nor hemolysis. To further characterize the behavior of red cells containing Hb Setif and to compare them with more familiar hemoglobinopathies, we have further defined the conditions required for pseudosickling. Suggestions are made concerning the nature of the process and the lack of clinical consequences.

PATIENTS

The compound heterozygote (FIK, II-1),³ his mother PEY, and his brother FER were studied. All were carriers of Hb Setif; FIK had

microcytic red cells (Coulter Counter) with target cells and hypochromia, as did his mother and brother to a lesser degree (Table 1), but Howell-Jolly bodies and siderocytes were not seen. The mother had been treated for menorrhagia and had had recurrent urinary tract infections with hematuria. Serum electrolytes, urea nitrogen, and creatinine were normal in all three patients, except for minimally elevated chloride in PEY (109 mmol/L). After overnight dehydration PEY was unable to concentrate her urine, but her sons were normal in this regard. Ophthalmologic examination (by Dr M. Hargrave) was normal in all three patients, except for a pigmented area in the temporal retina of FIK.

MATERIALS AND METHODS

For initial studies blood was obtained from the Setif/thalassemia compound heterozygote referred to earlier and a normal control. After collection in Sydney in ACD anticoagulant solution, the samples were shipped to Baltimore in ice and arrived within 48 hours with no evidence of hemolysis. Red cells were washed with 0.15 mol/L NaCl solution, gassed with carbon monoxide, and stored at -70°C until further use. Studies of sickling and oxygen affinity were done at Westmead Hospital, near Sydney, using blood collected in Na_2EDTA from three members of the affected family.

Pseudosickling was evaluated by incubating red cells in buffered potassium phosphate (4 to 5 mmol/L) or Tris HCl (0.01 mol/L) buffered NaCl or KCl solution for varying periods of time and at various temperatures. Osmolality was adjusted by alteration of NaCl or KCl concentration, and was measured with an Advanced Osmometer. After incubation, cells were fixed in 3.0% glutaraldehyde in phosphate buffered 0.15 mol/L NaCl solution pH 7.3. For estimating the proportion of deformed cells, samples were coded so that the observer did not know how they had been treated. All cells were counted by a single observer, and cells from different experiments (Table 2) were counted separately. Four hundred cells were counted and were considered as sickled only if they had the typical "sickled" shape (Fig 1). Crenated cells were also enumerated; it seemed likely that some of them represented transitional forms between discocytes and sickled forms, for their numbers decreased as

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Table 1. Carriers of Hb Setif

Sex	Age	Hb (g/dL)	MCV (fl)	Hb Setif %	MCHSC g/dL		Hb A ₂ %	Hb F %	Osmolarity Urine/Serum (mosm/kg/H ₂ O)	
					370	459				
PEY	F	52	10.0	80	14.3	4.9	5.7	1.3	0.6	277/290
FIK	M	28	13.2	65	39.5	12.6	14.0	2.8	0.5	1081/291
FER	M	26	14.0	78	16.4	5.7	6.6	1.9	0.7	1092/283

Abbreviation: MCHSC, mean corpuscular hemoglobin Setif concentration in buffer of given osmolality, calculated from hemoglobin, microhematocrit value, and proportion of Hb Setif.

Urine and serum osmolality were measured after overnight abstention from water.

Proportions of Hb Setif and A₂ were measured by elution from strips of cellulose acetate after electrophoresis at pH 8.6; 1.0% A₂ was seen in sample from FIK; but no "split" A₂ was seen in samples from PEY or FER. The % Hb Setif in FIK is higher than previously reported; his MCHSC in isotonic buffer (285 mosm/kg H₂O) was approximately 10.2 g/dL.

the proportion of clearly sickled cells increased. Apparent differences in sickling properties from day to day (compare FER in Table 2) were more likely due to variation in the observer than in the red cells. Red cell indices for cells suspended in hypertonic buffer were based upon automated (Coulter Counter) hemoglobin and red cell determinations and microhematocrits centrifuged for 15 minutes. Centrifugation for longer periods did not result in greater packing of the red cells. Cells were considered oxygenated when studied in aerated buffers; for deoxygenation the buffer was equilibrated with nitrogen, added to a N₂ filled tube, a drop of red cell suspension was added, additional N₂ was blown over the surface, and the tube was capped.

For purification of Hb Setif, CO-equilibrated buffers and hemolysates were used. Fractions were separated by chromatography on diethyl aminoethyl (DEAE)-Sephadex¹⁴ checked for purity by electrophoresis at pH 8.6, rechromatographed if necessary, and concentrated by ultrafiltration through cellophane membranes after regassing with CO. Concentrated (5 to 10 g/dL) purified solutions of Hb A or Setif were stored under CO at -70°C. In an attempt to measure solubility, samples of Hbs A and Setif were further concentrated overnight by ultrafiltration. A precipitate formed only in samples of Hb Setif and was isolated by centrifugation. To estimate the solubility of Hb Setif, the hemoglobin concentration of the supernatant was measured. Since solvent conditions were uncertain after ultrafiltration, a small amount of buffer was added to the precipitate, the specimen was mixed and equilibrated for several hours, and

Table 2. Sickling of Hb Setif-Containing Red Cells

Effect of Osmolarity				Effect of pH			Effect of Time of Incubation			
mosm/kg H ₂ O	FIK	FER	PEY	FIK	FER	PEY	Time (H)	FIK	FER	PEY
285	23	0	0	6.2	4	0.2	0.5	6	0.5	0.5
331	46	3	0.5	6.6	11	1	1	17	1	0.2
370	64	5	5	7.0	19	2	1.5	24	2	0
393	78	17	4	7.5	44	2	2	41	2	2
459	93	31	14	7.8	65	3	4	59	5	6
494	93	36	19				6	87	16	17
							8	95	47	31
							10	95	66	47
							12.5	97	54	—
							23	96	91	—

NOTE. Cells incubated at 23°C in 0.04 mol/L PO₄/NaCl buffer: 24 hours, pH 7.3; 459 mosm/kg, pH 7.3; two hours, 436-451 mosm/kg H₂O. See text for description of patients FIK, FER, and PEY.

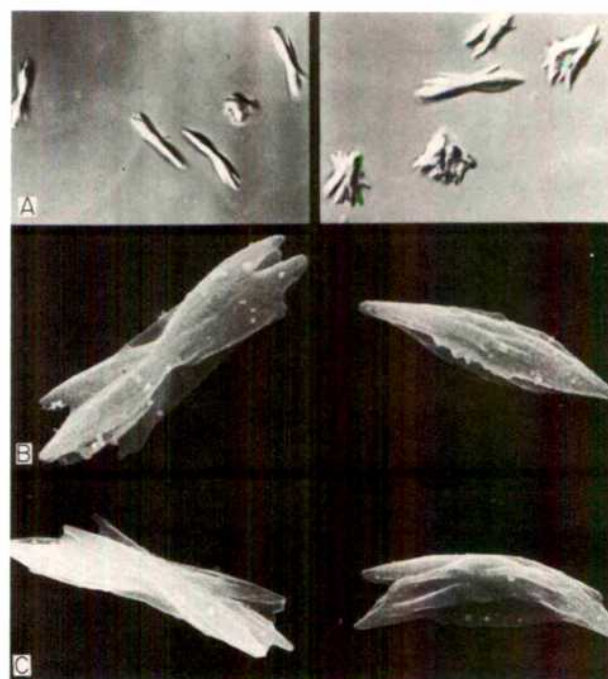


Fig 1. Pseudosickling of Hb Setif-containing red cells. (A) Light microscopy, Nomarsky optics. (B, C) Scanning electron microscopy.

recentrifuged; the process was repeated once again, and hemoglobin concentrations of the supernatant fluids were also measured.

These experiments yielded relatively large crystals of Hb Setif, but attempts to estimate solubility of the abnormal hemoglobin yielded results that were erratic and unreproducible. More satisfactory measurements were obtained using a different technique. Concentrated specimens of purified hemoglobin A or Setif (5 to 10 g/dL) were dialyzed overnight at 5°C against 5 mmol/L potassium phosphate in 150 mmol/L KCl (pH 7.2 unless specified otherwise) saturated with carbon monoxide. Dialyzed solutions were diluted with buffer to a chosen hemoglobin concentration (usually 4 g/dL), centrifuged, and regassed with carbon monoxide. These solutions were added to Centricon-10 (Amicon Corporation, Scientific Systems Division, Danvers, MA) filters (10 kd nominal cutoff). Each concentrator was filled with 2 mL of hemoglobin and then centrifuged at 6,000 rpm (RCF = 4,340 g) in a Sorvall SS-34 rotor at 5 to 10°C. In most experiments, the centrifuge was stopped at five-minute intervals, the hemoglobin solutions above the filters were mixed, and centrifugation was resumed. At 15- to 20-minute intervals the hemoglobin solutions above the filters were removed from the concentrators and centrifuged at 10,000 rpm (RCF = 12,100 g) for ten minutes to sediment any precipitate. Supernatant fluid was decanted, a 15 to 25 µL sample was removed for measurement of hemoglobin concentration, and the remainder was returned to the concentrator and the process repeated until the volume of supernatant fluid was too small (about 0.2 mL) to be handled (typically, 6 or 7 cycles). In such experiments if the supernatant hemoglobin concentration remained more or less constant with repeated centrifugation (eg, Hb Setif in Fig 2B), that concentration was considered as the "solubility" of the hemoglobins. If the concentration rose continuously, one could only say that "solubility" exceeded the final concentration achieved. If one started with a dilute solution (Fig 2A), the final concentration achieved might well be less than the solubility.

For experiments involving deoxyhemoglobin, carboxyhemoglobin

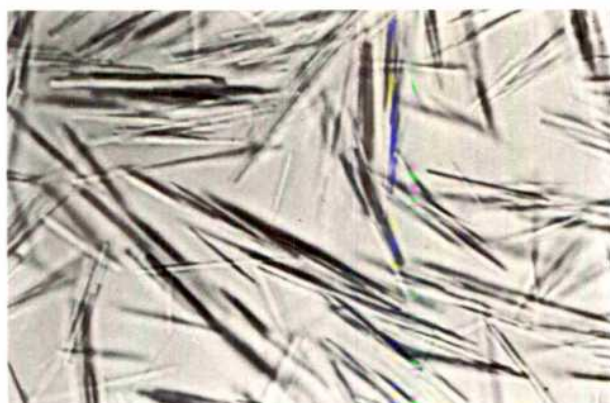


Fig 2. Centrifugation of purified solutions of Hb A and Setif in 0.15 mol/L KCl, 0.005 mol/L PO_4 , pH 7.2, over Centricon filters. (A) Initial concentration 0.5 g/dL; (B) Initial concentration 4 to 5 g/dL.

was first converted to the oxy form by placing it in an IL 237 Tonometer (Instrumentation Laboratories, Inc, Lexington, MA) and exposing it to oxygen and light from a 200-watt bulb for 30 minutes at 5°C. The oxyhemoglobin was then converted to the deoxy form by an additional 30-minute exposure to nitrogen. Most of the sample became deoxygenated (84% to 92%) with negligible methemoglobin production (<2.4%). Each concentrator was filled with 1.5 mL of the deoxygenated solution, a few crystals of Na dithionite were added, and the solution was overlaid with a small amount of mineral oil. The concentrators had been preflushed with nitrogen, and they were enclosed in 50-mL centrifuge tubes that also had been flushed with nitrogen. Centrifugation of the concentrators was not interpreted at five-minute intervals but was allowed to proceed continuously for 40 minutes.

To study interaction between Hb A and Setif, samples of the purified carboxyhemoglobins were mixed to give proportions of Hb Setif from 10% to 50%, and were subjected to ultrafiltration, essentially as described above. The proportions of Hb A and Setif in the supernatants were determined after elution from cellulose acetate strips. Somewhat higher values were obtained by densitometry after electrophoresis on agar gel.¹⁵

Hemoglobin concentrations were determined by conversion to cyanmethemoglobin with Drabkin's reagent. Percentages of oxyhemoglobin, carboxyhemoglobin, and methemoglobin were determined using an IL 282 CO-Oximeter (Instrumentation Laboratories). Deoxyhemoglobin was taken as the sum of these three subtracted from 100. Oxygen dissociation curves were constructed by equilibrating blood with gas mixtures containing 5% CO_2 , nitrogen, and oxygen. Saturation was measured with the CO-Oximeter, and pH and PO_2 with an IL-1312 blood gas analyzer (Instrumentation Laboratories).

RESULTS

Pseudosickling (Fig 1) was increased by increased osmolality (Table 2) and was greater at 37°C than at 23°C. None was seen after 20 hours incubation at 0°C. There was little difference between tris or phosphate buffers, and there was not much difference if cells were suspended in NaCl or KCl (data not shown). Sickling was enhanced in alkaline buffer, and decreased in acid (Table 2). Although MCHC increased promptly when cells were suspended in hypertonic buffer (within an hour), sickling did not become marked until several hours had passed (Table 2). Consistently, FIK

showed more marked sickling and PEY less; the concentration of Hb Setif within their red cells was highest in FIK and lowest in PEY (Table 1). Very little sickling (which could not be distinguished from crenation) was seen if cells were deoxygenated before incubation.

The sediment collected after overnight ultrafiltration of purified Hb Setif contained long birefringent needle-like crystals (Fig 3). As noted in "Methods," Hb A did not precipitate under the conditions. The sediment after brief ultracentrifugation in Centricon tubes contained tiny clusters of birefringent needles that readily redissolved in buffer.

When dilute (<1 g/dL) solutions of carboxy Hbs A or Setif were centrifuged on Centricon-10 filters, hemoglobin concentration above the filter rose from <1 to 7 to 8 g/dL (Figure 2A). Trace amounts of precipitate formed in samples containing Hb Setif. When the starting solution was more concentrated (Fig 2B), a difference became evident. The hemoglobin concentration of samples containing Hb A rose steadily, exceeding 30 g/dL; considerable amounts of precipitate formed in samples containing Hb Setif at concentrations above 5 g/dL, and the supernatant hemoglobin concentration rose very slowly. It should be emphasized that equilibrium was never achieved in these experiments, and the "solubilities" described below represent differences in the behavior of Hb A and Setif rather than absolute numbers.

Since it seemed likely that a concentration gradient formed above the filter during a 15- to 20-minute "run" (the concentration being highest immediately above the filter), experiments were repeated, stopping the centrifuge every five minutes, mixing the supernatant fluid, and resuming centrifugation. Little difference was seen in comparison with earlier experiments. When precipitates were redissolved and the experiment repeated, results were similar, suggesting that the process observed was reversible precipitation rather than denaturation. Experiments were repeated after dialyzing samples to pH 6.6 or 7.5 (data not shown). There was no difference between the two samples of Hb A (27 g/dL final

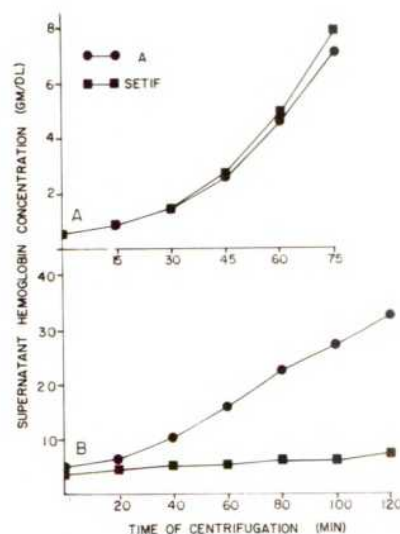


Fig 3. Crystals of Hb Setif collected after overnight ultrafiltration of a purified solution through a cellophane membrane.

concentration), but Hb Setif was more soluble at the lower pH (11 v 7 g/dL).

During centrifugation in the presence of Na dithionite under mineral oil, the concentration of "deoxy" Hb Setif continued to rise (final concentration 13.3 g/dL), while the concentration of carboxy Hb Setif rose more slowly (5.2 g/dL final concentration). No difference was seen between samples of deoxy and carboxy Hb A treated in the same fashion: the concentration of both continued to rise, and final concentrations were similar (10.7 v 10.0 g/dL). In a second experiment using more dilute samples, very similar results were obtained (8.4 v 3.9 g/dL for Hb Setif, 7.8 v 6.4 g/dL for Hb A).

A crude hemolysate was prepared, dialyzed against buffered KCl, and concentrated by centrifugation as described above. No precipitate formed up to a concentration of about 10 to 15 g/dL, but small amounts appeared thereafter. Loss of hemoglobin through precipitation (about 40% of the initial amount) was evident when total hemoglobin remaining above the filter was estimated from supernatant volume. To study the behavior of hemolysates in greater detail, artificial mixtures of Hb A and Setif were ultrafiltered. There was little difference between samples containing 10% and 23% Setif (Fig 4). The concentration of samples initially containing 36% rose more slowly, and those containing 50% Hb Setif still more slowly due to loss of hemoglobin that precipitated on the filters as the hemoglobin concentration of the overlying solution increased. The precipitates were composed of both hemoglobins A and Setif (Table 3) but were relatively enriched in the latter compared to the composition of the supernatant mixture. The solubility of Hb Setif in the solutions (which were not in equilibrium with precipitates) was approximately 6 g/dL (total hemoglobin concentration 13.5 to 27.3 g/dL, % Hb Setif 22% to 49%).

Oxygen affinity was measured four to five days after blood was collected. Fresh blood from a normal control (SC) had virtually the same p_{50} at pH 7.4 (28.5 mm Hg) as that stored for four days (27.5, 29.5). FIK had minimally higher affinity than FER (27.5, 27 v 29.0, 28), but there was no suggestion

that FIK's curve diverged from that of the control at high (68%) or low (8%) saturation.

DISCUSSION

Hb Setif, an alpha chain variant, usually comprises 12% to 15% of hemolysates,^{3,12,13} reflecting the usually low proportion of alpha chain variants and perhaps the instability of the abnormal hemoglobin.¹² The high proportion in the blood of FIK (39.5%) reflects the coexistence of one or more thalassemia genes,³ as could be true for higher proportions observed in Saudi Arabian patients.¹¹ The lower proportion in PEY's blood (in comparison to FER) may reflect coexisting iron deficiency.

The most remarkable property of Hb Setif is its capacity to induce sickling of red cells in vitro. "Sickling" has been described in human red cells containing Hb I,¹⁶⁻¹⁸ Barts,¹⁹ and A,¹⁷ in addition to those containing Hb S, and crystals have been described in red cells containing Hb C. The initial patient with Hb I- α thalassemia was a compound heterozygote whose red cells contained 70% Hb I¹⁶; clinically she exhibited no vaso-occlusive manifestations, her fresh red cells were not sickled, and the rheologic properties of her blood were unremarkable. Sickling of Hb Barts has been described only once, and that induced in normal red cells is a laboratory curiosity. Red cells containing crystals of Hb C can be found in freshly collected blood,²⁰ and crystallization can be induced readily if the cells are incubated in 3% NaCl solution.²¹ Vaso-occlusive crises do not occur, but decreased deformability of the red cells has been implicated in the genesis of hemolytic anemia. Hb C is less soluble than Hb A, but the difference is not striking (35 v >46 g/dL²¹).

Red cells of deer,²²⁻²⁴ some sheep,²⁵ some mongooses,²⁶ and other species^{27,28} can be induced to undergo sickling-like changes in vitro. Deoxygenation of the sample is not required; other factors involved include high pH,^{24,29} the presence of EDTA,^{26,27} and in vitro dehydration.²⁶ Only in angora goats are distorted red cells thought to exist in vivo^{28,30} and there polymerized hemoglobin was not found in freshly collected cells. Efforts to produce in deer disability related to the sickling phenomenon have been unsuccessful.^{30,31}

Our experiments with Hb Setif here were carried out with concentrated ("nonideal") solutions. Results are difficult to interpret because a significant portion of the solution is occupied by the solute, and the properties of the solute hemoglobin as a polyelectrolyte may alter its behavior. Equilibrium conditions probably were never achieved, and the "solubilities" recorded are only semiquantitative. With those reservations we interpret our data to show that Hb Setif is much less soluble than hemoglobin A, that mixtures of Hb A and Setif also show decreased solubility, and that conditions that raise the intracellular hemoglobin concentration foster pseudosickling. Of some interest, solubility is lower and (as in deer) sickling is enhanced at pH above 7.4 (Table 2).

The site of amino acid substitution in Hb Setif is an important contact point between α and β chains in the oxy

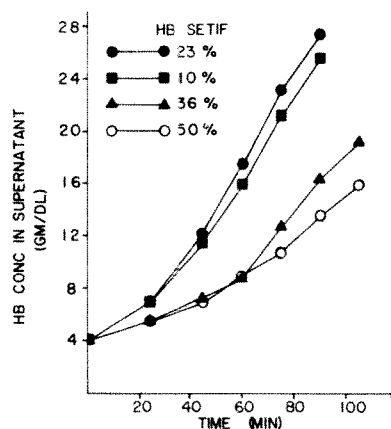


Fig 4. Centrifugation of artificial mixtures of CO Hb A and Setif in 0.15 mol/L KCl, 0.005 mol/L PO_4 , pH 7.2, over Centricon filters.

Table 3. Composition of Artificial Mixtures of Hb A and Setif During Ultrafiltration

Time (min)	Initial Composition of Mixtures								
	23% Hb Setif			36% Hb Setif			50% Hb Setif		
	Hb Concentration Solution g/dL	% Setif		Hb Concentration Solution g/dL	% Setif		Hb Concentration Solution g/dL	% Setif	
		Solution	Ppt		Solution	Ppt		Solution	Ppt
25	7.0	26	—	5.5	37	—	5.4	49	—
45	12.1	26	—	7.3	37	—	6.9	47	—
60	17.5	26	—	9.5	36	—	8.7	48	63
75	23.1	25	33	12.6	38	—	10.7	47	—
90	27.3	22	29	16.3	36	48	13.5	49	60
105	—	—	—	19.1	35	43	15.9	47	52

NOTE. Carboxyhemoglobin solutions of initial concentration 4 g/dL, in 5 mm PO₄ 0.15 mol/L NaCl, pH 7.2 were subjected to ultrafiltration through Centricon-10 filters (see text).

Abbreviation: Ppt, precipitate.

conformation. Loss of the contact should reduce oxygen affinity; contrary to expectation, oxygen affinity is only slightly perturbed, but mild instability is produced.¹² The nature of the substitution, tyrosine for aspartic acid, might be expected to produce further anomalies because the side chains of the two amino acids differ in size and polarity.¹³ Aubert et al¹² suggested that the difference produces a change in tertiary structure of the α chain in the vicinity of the substitution and suggested that the normal $\alpha_1\beta_2$ contact within the molecule might be replaced by contacts between α chains in adjacent molecules. Such contacts could explain self-association into crystals, if the sites on the molecules were dissimilar, but it seems more likely that the change in tertiary structure is more widespread than they proposed, and that virtually all portion(s) of the external surfaces of the molecule could be involved.

Our observation that precipitates from mixtures of Hb A and Setif always yielded Hb A on analysis could reflect entrapment of the latter hemoglobin. No attempt was made to wash the precipitates because of erratic results obtained when washing precipitates of pure Hb Setif (see "Methods"). The magnitude of the apparent interaction under certain conditions (70% Hb A, Table 2) suggests that mixed tetramers ($\alpha^A\alpha^{\text{Setif}}\beta_2$) may be important units in crystals within red cells, as they are in polymers forming in mixtures of hemoglobin S with other hemoglobins.⁸⁻¹⁰ Existing as mixed tetramers in the crystal, they would be expected to reassociate into hemoglobins A and Setif during electrophoresis.

Edelstein et al³³ recently suggested that self-association of Hb Setif, when oxygenated, could in turn affect oxygen binding. By analogy to Hb S, which associates in the T (deoxy) conformation and has anomalously low affinity in concentrated solution, one would expect self-association of Hb Setif (in the R or oxy conformation) to increase affinity. As with Hb S, the difference might only be present at high hemoglobin concentration (ie, red cells or artificially prepared solutions of the pure hemoglobin). Raik et al found normal affinity of FIK's blood,³ and we found a minimal difference from normal, but further studies at varying concentrations of the purified abnormal hemoglobin would seem indicated.

At the temperature of the centrifuge (5°C to 10°C) Hb

Setif began to precipitate (crystallize?) from crude hemolysates at Hb Setif concentrations about 6 g/dL and precipitated from solutions containing 25% Hb Setif at a total hemoglobin concentration of 23 g/dL (Fig 4), well below the MCHC of red cells. If blood from the three patients studied is compared at equal sickling (about 20%, Table 2) at 23°C, the intracellular Hb Setif concentration in those cells is similar (5.7, 5.7 and less than 10.2 g/dL, Table 1), despite the difference in temperature.

Pseudosickled cells appear to contain bundles of twisted fibers (Fig 1, and Fig 28 in ref. 4). Those fibers (and earlier stages of molecular aggregation) probably increase red cell rigidity, and one might expect to find infarctions in the hypertonic renal medulla similar to those seen in sickle cell disease or trait. Neither FIK nor FER showed evidence of such infarctions; hematuria and hyposthenuria in their mother was considered by her physician to be a consequence of recurrent infection. Absence of lesions could reflect a difference in solubility due to the difference in temperature between our in vitro studies (5°C to 23°C) and 37°C, but it seems more likely that the lag between red cell shrinkage and sickling (Table 2) may account for absence of clinical findings: erythrocytes probably escape from the vasa recta before significant numbers of polymers or crystals have formed. A similar argument has been used to explain absence of lesions from vascular beds with a very low PO₂ (ie, the myocardium) in sickle cell anemia, but "delay times" in that disease³⁴ are probably much shorter than the lag times seen here.

ACKNOWLEDGMENT

Dr Wilbur Hughes gave us the use of his laboratories at the Institute of Clinical Pathology and Medical Research at Westmead Hospital; we are most grateful to him and his staff for their friendliness and cooperation. We wish to thank Dr M. Hargrave for examining the patients' eyes, Claire Norwood for measuring the proportion of Hb Setif in a large number of samples, the laboratory of Dr Ronald Nagel of the Albert Einstein College of Medicine for scanning electron microscopy, and Dr Gary Ackers for helpful discussion. We are indebted to Dr F. Drupt, who provided us with a copy of his doctoral thesis; the insights it provided were most useful in the present work.

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Clinical Experience With Transfusion of Leukocyte-Poor Platelet Concentrates Prepared by Filtration With Prostacyclin

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Repeated transfusions with platelets from randomly selected donors lead to HLA alloimmunization in about 50% of patients due to lymphocyte contamination of platelet concentrates. Attempts to remove the leukocytes from the platelet concentrates by additional centrifugation steps led to substantial loss of platelets. We report a new procedure for removal of almost all leukocytes with excellent platelet recoveries. Single donor concentrates are treated with 50 ng/mL prostacyclin to inactivate the platelets transiently. The concentrates are then passed through a cellulose-acetate filter to remove the leukocytes. In 30 concentrates this treatment reduced the contamination by leukocytes to

less than 0.1 million per concentrate with a platelet recovery of $89\% \pm 1\%$ (mean $\pm$ SEM). Thirty filtered platelet concentrates transfused to ten thrombocytopenic patients within one hour after filtration were well tolerated and led to corrected count increments of $(22.0 \pm 1.1) \times 10^6$ /mL blood after one hour and normal survival thereafter. In four of five patients these concentrates reduced the bleeding time. We conclude that transient inactivation of platelets by prostacyclin enables optimal removal of leukocytes and may help to reduce alloimmunization during frequent transfusions with platelet concentrates.

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IN THE LAST few years chemotherapy for patients with acute leukemia has been intensified. This has rapidly increased the need for platelet concentrates as part of the supportive therapy. One of the major problems associated with repeated platelet transfusions is the development of refractoriness, primarily due to HLA alloimmunization.¹⁻³ Only in a minority of the cases are platelet-specific antibodies triggered, which seldom form a problem for platelet transfusions.^{4,5} There is increasing evidence that HLA alloimmunization is induced by the contaminating lymphocytes, and clinical studies have shown that transfusion of leukocyte-poor blood components reduces the incidence of HLA alloimmunization.⁶⁻⁸ At present pooled⁷ or single donor⁸ platelet concentrates are depleted from contaminating leukocytes by additional centrifugation, which reduces the contamination with leukocytes to 0.8 to 2.2×10^8 cells per concentrate. Unfortunately this method leads to a 30% loss of platelets. More leukocytes can be removed by increasing the centrifugation speed, but this treatment further reduces the platelet yield. An alternative method is filtration which has been successfully applied to remove leukocytes from whole blood, but most of the platelets stick to the filters and the loss of platelets is about 80%.⁹

We here report a new procedure to prevent platelet adherence to the filter by inactivating the platelets with prostacyclin. This treatment does not interfere with the removal of leukocytes and results in platelet recoveries of about 90%. Preliminary data show that these platelets recover their hemostatic properties after transfusion.

MATERIALS AND METHODS

Preparation of platelet suspensions for in vitro studies with prostacyclin. The transient inactivation of platelets by prostacyclin was investigated in platelet-rich plasma (PRP) and in single donor platelet concentrates. For the preparation of PRP, venous blood was collected in citrate (0.1 vol of 130 mmol/L sodium citrate) from healthy donors who claimed not to have taken any medication for at least ten days. The blood was centrifuged (120 g, 10 minutes, at room temperature), and the supernatant, PRP, was collected and standardized at 2.5×10^8 platelets/mL by dilution in platelet-poor plasma (PPP).

Single donor platelet concentrates were prepared by cytopheresis with a Haemonetics V50 using ACD-A as anticoagulant. In 30 preparations with a volume of 288 ± 7 mL (mean $\pm$ SEM) the platelet yield was $(4.1 \pm 0.2) \times 10^{11}$ with a contamination of $(3.6 \pm$

$0.5) \times 10^8$ leukocytes. For in vitro studies samples were taken from the concentrates and standardized at 2.5×10^8 platelets/mL by dilution with autologous plasma. The platelet suspensions were treated with different concentrations of prostacyclin, as indicated in the "Results" section. Prostacyclin (PGI₂, Epoprostenol, The Wellcome Foundation Ltd, London) was approved for clinical use. The content of one vial (0.5 mg) was dissolved in 10 mL 0.01 N NaOH, distributed over 20 ampules and stored at -80°C .

Preparation of filtered platelet concentrates. Filtration was performed with cellulose acetate filters (Erypur or Cellselect, Organon Teknika, Amsterdam, The Netherlands). Just before filtration the filters were rinsed with citrate buffer (sodium citrate, 29.9 g/L, anhydrous citric acid, 563 g/L, 2 g/L human albumin, pH 6.5). The prostacyclin solution was thawed and immediately added to the platelet concentrates to obtain a final concentration of 50 ng/mL (0.1 mL of stock solution per 100 mL of platelet concentrate). No adjustments were made for the minor differences in platelet concentration. The concentrates were then passed through the filter at a flow rate of 10 mL/min, and residual platelets in the filter were collected with 200 mL of additional citrate buffer. The recovery after the filtration procedure was measured by counting the leukocytes visually in a Burkert chamber and the platelets in a Baker 810 Trombocounter (Baker, Allentown, PA). The number of leukocytes after filtration was usually extremely low and was therefore measured after concentrating a 20-mL suspension by centrifugation (1000 g, 20 minutes, at room temperature) and resuspension in 0.5 mL of plasma. The filtered concentrates were used for transfusion without prior removal of the prostacyclin.

Aggregation studies. Platelet aggregation was measured in a dual channel aggregometer (Payton Ass, Scarborough, Canada) at a stirring speed of 900 rpm at 37°C following stimulation with 5 μg /mL of collagen (Hormon Chemie, Munchen, FRG).

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Transfusion of Filtered Platelet Concentrates. The filtered platelet concentrates were administered to ten patients on a prophylactic basis. Nine patients were treated for acute myeloid leukemia, and one patient was treated for a blast crisis of chronic myeloid leukemia. All patients were profoundly thrombocytopenic (platelet counts $<15 \times 10^6/\text{mL}$) but otherwise clinically stable. Platelet counts were obtained before and one hour after transfusion and every day thereafter. Post-transfusion results were expressed as corrected count increments (CCI) according to the formula:

$$\text{CCI} = \frac{\text{absolute increment} \times \text{body surface area (m}^2\text{)}}{\text{number of transfused platelets} \times 10^{11}}$$

In five patients who showed post-transfusion counts of more than $50 \times 10^6/\text{mL}$ bleeding time measurements were performed by means of the Simplate II method (General Diagnostics, Division of Warner-Lambert, Morris Plains, NJ).

RESULTS

Inhibition of platelet function in vitro by prostacyclin. In an initial series of experiments with PRP the optimal concentration of prostacyclin was sought which induced complete inhibition of platelet function for at least one hour but which also permitted the platelets to recover thereafter. Platelet function was measured as the maximum aggregation in response to collagen and was expressed as a percentage of control (without prostacyclin). Figure 1 illustrates that between 1 and 20 ng/mL prostacyclin completely prevented aggregation. The inhibition was transient, and platelet function rapidly recovered upon addition of 1 ng/mL, whereas much more time was required for spontaneous recovery with higher doses of prostacyclin. Thus a dose of 5 ng/mL of prostacyclin was considered optimal for inhibition of platelet function for at least one hour. Single donor platelet concentrates contained about five times more platelets per volume, and initially the dose of prostacyclin was increased to 25 ng/mL, but we found that twice as much was required for the desired degree of inhibition (Fig 1).

The time required to recover from the prostacyclin inhibi-

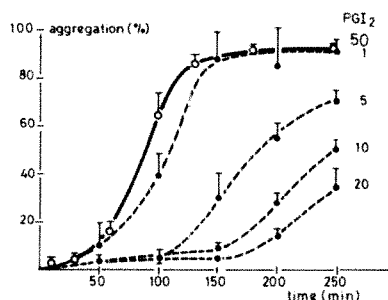


Fig 1. Transient inhibition of collagen-induced aggregation by different doses of prostacyclin (ng/mL, final concentrations). Aggregation induced by $5 \mu\text{g/mL}$ of collagen was measured as maximal change in light transmission and expressed as a percentage of a simultaneously run control (no prostacyclin added). The response of PRP (broken lines, 2.5×10^8 cells/mL) was compared with that of platelets collected from single donor concentrates (solid line, containing 50 ng/mL prostacyclin) after dilution in autologous PPP to a final concentration of 2.5×10^8 cells/mL (mean $\pm$ SEM, $n = 6$).

tion could be greatly shortened by centrifugation and resuspension of the platelets in prostacyclin-free medium. In PRP this treatment abolished the effect of up to 20 ng/mL prostacyclin within ten minutes (Fig 2A), whereas in platelet concentrates inactivated with 50 ng/mL prostacyclin the ability to aggregate was restored after 45 minutes (Fig 2B).

Filtration of platelet concentrates. Single donor platelet concentrates were treated with 50 ng/mL prostacyclin within two hours after preparation and were immediately passed through the filter. The number of platelets per concentrate fell from $(4.0 \pm 0.2) \times 10^{11}$ before filtration to $(3.6 \pm 0.2) \times 10^{11}$ thereafter, resulting in a yield of $89\% \pm 1\%$ (mean $\pm$ SEM, $n = 30$). The contamination of leukocytes fell from $(3.6 \pm 0.5) \times 10^8$ before filtration to less than 0.1 million cells per concentrate thereafter, which equals a depletion of more than 99.9%.

Transfusion of filtered platelet concentrates. A total of 30 filtered concentrates was administered at a flow rate of 10 mL/min to ten patients with leukemia. The transfusions were carried out within one hour after filtration when most of the platelets were still inactive. Pulse rate and BP were measured once every ten minutes and remained normal. No other side effects were observed. Table 1 lists the post-transfusion count increments (CCI). Transfusion of filtered

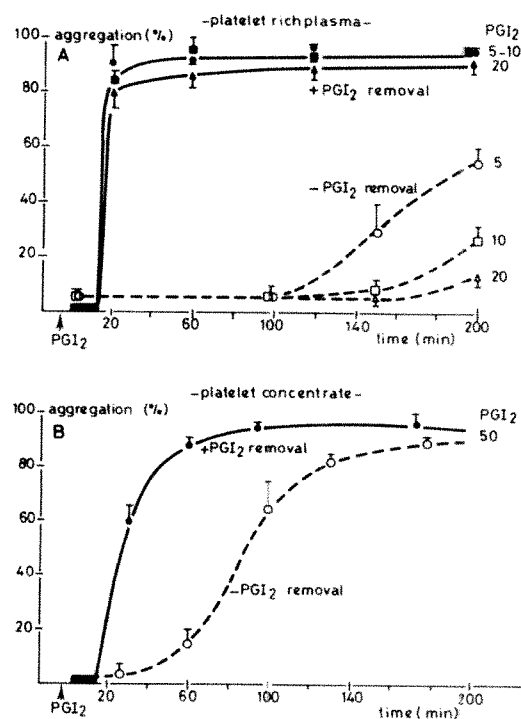


Fig 2. Rapid recovery of aggregability of prostacyclin-inactivated platelets by centrifugation/resuspension in plasma. PRP was treated with different doses of prostacyclin (5 minutes, 22°C) and subsequently pelleted and resuspended in prostacyclin-free, autologous plasma. The recovery of collagen ($5 \mu\text{g/mL}$)-induced aggregation was expressed as a percentage of control (Fig 2A, solid lines) and compared with the spontaneous recovery without prostacyclin removal (Fig 2A, broken lines). Figure 2B illustrates similar experiments using platelets obtained from single donor concentrates (mean $\pm$ SEM, $n = 4$).

Table 1. Post-transfusion Platelet Counts

Time After Transfusion	Increment ($\times 10^6/\text{mL}$)	
	Absolute	Corrected
1 h	40.1 $\pm$ 2.5	22.0 $\pm$ 1.1
18-24 h	28.5 $\pm$ 2.0	14.8 $\pm$ 0.8
42-48 h	13.0 $\pm$ 1.3	7.2 $\pm$ 0.7

NOTE. Mean $\pm$ SEM; n = 30.

concentrates resulted in CCIs of 22.0 and 14.8 $\times 10^6/\text{mL}$ blood at one hour and between 18 and 24 hours after transfusion respectively. For comparison, 26 transfusions of single donor HLA-matched platelets, administered to a comparable group of patients, produced a mean corrected count increment at one hour of 22.1 $\pm$ 2.8 $\times 10^6/\text{mL}$ and a mean corrected count increment at 24 hours of 10.7 $\pm$ 1.2 $\times 10^6/\text{mL}$. Five patients were transfused with filtered platelet concentrates during the thrombocytopenic phase of their remission induction therapy, and none of the patients showed signs of hemorrhage. Bleeding times were performed in these five patients when post-transfusion counts were more than 50 $\times 10^6$ platelets/mL blood. In four patients a significant reduction of the bleeding time was observed (Table 2).

DISCUSSION

For many years blood filters have been used to remove leukocytes from whole blood. The best results were obtained with cellulose-acetate filters and cotton-wool filters, both leading to a leukocyte depletion of about 98%.⁹ Unfortunately these filters also removed most of the platelets and were therefore unsuitable for removing leukocytes from platelet concentrates. The present study shows that the loss of platelets during filtration can be reduced to a minimum by transiently inactivating these cells with prostacyclin. Currently platelet concentrates are best depleted from leukocytes by additional centrifugation. Such a procedure takes about 15 minutes per concentrate and is therefore faster than our filtration technique, which takes about twice as long. The great advantage of our filtration procedure lies in the much better platelet recovery and the almost complete removal of leukocytes, resulting in concentrates that may be optimally effective for preventing HLA alloimmunization after repeated platelet transfusions. One may expect that these concentrates will rescue the need for HLA-matched platelets, which will make up for the cost of the filters and will

even be more economical. Although the prostacyclin treatment appears successful in *in vitro* studies, two questions must be addressed before the method can be applied clinically: (1) How safe is the use of prostacyclin, which is known in certain conditions to induce hypotension? and (2) Are the transfused platelets hemostatically effective? Prostacyclin has been successfully applied in patients with peripheral vascular disease,¹⁰ chronic renal disease,¹¹ and thrombotic thrombocytopenic purpura.¹² In a recent study in normal volunteers, clinical tolerance, inhibition of platelet aggregation, and intracellular levels of cyclic AMP were compared during infusions of prostacyclin at rates up to 15 ng/kg/min.¹³ For short-term infusions (up to 60 minutes) the maximum tolerated rate was 10 ng/kg/min. The increase of cyclic adenosine monophosphate levels and inhibition of the circulating platelets were transient and returned to pretreatment values within 30 minutes after the end of infusion. In our study filtered platelet concentrates were transfused at a rate of 10 mL/min. Assuming that all prostacyclin added to the platelets prior to filtration was still effective at the time of transfusion, this would equal an infusion rate of about 6 ng/kg/min, which is well below the maximum tolerated rate. Indeed, no signs of hypotension or other side effects were observed. The second question is more difficult to answer. Our *in vitro* studies show that the prostacyclin-treated platelets regained the ability to aggregate in response to collagen after a few hours incubation at room temperature. This recovery period could be greatly shortened by pelleting the cells and resuspending them in prostacyclin-free medium. Although this provides a means to reactivate the platelets prior to infusion, we chose to transfuse the inactive platelets. Apart from bypassing a time-consuming washing step, this procedure protected the platelets optimally against *in vitro* activation and enabled them to recover in the blood stream. Probably the dilution *in vivo* in the circulating blood mimics the situation *in vitro* when the cells are resuspended in fresh plasma, enabling the platelets to become hemostatically active. Our *in vivo* data appear to support this concept and show a reduction in bleeding time in four of five patients following transfusion of prostacyclin-treated platelets. Obviously the question of whether transient inactivation *in vitro* improves the hemostatic functions *in vivo* requires a more detailed study.

The ultimate goal for the preparation of leukocyte-depleted platelet concentrates is to prevent HLA alloimmunization in patients who are frequently transfused with platelets. Recent studies by Murphy et al⁸ have already revealed a reduction from 48% to 16% in alloimmunization when the leukocyte contamination was kept below about 2×10^8 per concentrate. With our technique the contamination is less than 1×10^5 leukocytes per concentrate, and a further reduction in alloimmunization may be expected. Thus the use of prostacyclin-treated, filtered platelet concentrates may reduce the incidence of the troublesome febrile transfusion episodes that are inherent to current transfusion regimes, prolong the time when random donor concentrates are effective, and reduce the need for HLA-matched donor platelets.

Table 2. Simplex Bleeding Times Before and After Transfusion of Filtered Platelet Concentrates

Patient	Platelet Count ($\times 10^9/\text{mL}$)		Bleeding Time		
	Before	After	Before	After I	After II
1	15	51	>30'	17'10"	15'30"
2	5	71	>30'	25'30"	18'30"
3	9	61	>30'	15'10"	15'40"
4	8	55	>30'	>30'	27'10"
5	9	59	>30'	18'20"	18'40"

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Hypodiploidy Is Associated With a Poor Prognosis in Childhood Acute Lymphoblastic Leukemia

By Ching-Hon Pui, Dorothy L. Williams, Susana C. Raimondi, Gaston K. Rivera, A. Thomas Look, Richard K. Dodge, Stephen L. George, Frederick G. Behm, William M. Crist, and Sharon B. Murphy

Leukemic cells from 31 (7.6%) of 409 children with newly diagnosed acute lymphoblastic leukemia (ALL) had a hypodiploid karyotype. The patients' ages ranged from 0.8 to 17 years (median, 5 years) and their initial leukocyte counts from 1.0 to $132 \times 10^9/L$ (median, $12.7 \times 10^9/L$). Modal chromosome numbers for the leukemic stem lines were 45 in 26 cases, 28 in two cases, and 26, 36 and 43 in one case each. Seven cases had one to three additional abnormal lines due to clonal evolution. Chromosome 20 was lost most frequently (nine cases). Structural abnormalities—including chromosomal translocations (21 cases), deletions (ten cases), duplications (two cases), or inversions (one case)—were common findings; the nonrandom translocations consisted of the $t(1;19)(q23;p13.3)$ in

two pre-B cases and $tdic(9;12)(p171;p172)$ in three cases of common ALL. When compared with hyperdiploid cases (>50 chromosomes), ALL with hypodiploidy was found to have a poorer outcome and was more likely to be associated with chromosomal translocations, higher serum lactic dehydrogenase levels, and age <2 or ≥ 10 years. Moreover, patients with hypodiploid ALL fared as poorly as those with pseudodiploid karyotypes, even though their leukocyte counts and serum lactic dehydrogenase levels were lower and they had a comparable frequency of leukemic cell translocations. Hypodiploidy is therefore an unfavorable karyotypic feature in childhood ALL.

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CHROMOSOMAL abnormalities have attracted much recent attention as independent prognostic factors in childhood acute lymphoblastic leukemia (ALL). The first clinically relevant subgroup to be described had hyperdiploid stem lines with >50 chromosomes per leukemic cell and comprised about 30% of children with ALL, a subset that has proved to have the most favorable prognosis.¹⁻⁴ Subsequent studies have shown that the pseudodiploid karyotype, defined by 46 chromosomes with structural alterations, is associated with the poorest response to therapy.²⁻⁷ Hypodiploidy, by contrast, is a relatively uncommon finding, affecting only 3% to 9% of patients with ALL,²⁻⁸ and its prognostic significance has been controversial. Some investigators suggest that hypodiploidy at diagnosis is a favorable risk feature,^{1,5,7} while others have shown it to be associated with a poor treatment outcome.^{4,8} In this study, which includes the largest number of banded cases of ALL reported to date, we demonstrate that leukemic cell hypodiploidy is associated with a poor prognosis.

MATERIALS AND METHODS

Of 710 consecutive children with newly diagnosed ALL who were admitted to St. Jude Children's Research Hospital from November 1978 to October 1986, 421 had complete G-banding of all chromosomes from bone marrow leukemic cells. Twelve of these patients had B cell ALL and were treated separately. The present study was restricted to the 409 patients with non-B ALL who had completely banded leukemic cell chromosomes to avoid possible underrepresentation of clonal abnormalities. These children were enrolled in three Total Therapy studies⁹⁻¹¹ after informed, written consent was obtained. The investigations were approved by the institution's clinical trials review committee.

Cytogenetic analysis. Bone marrow samples were processed according to the method of Williams et al.¹² Marrow cells were collected in RPMI 1640 medium containing 30% fetal calf serum with added L-glutamine and antibiotics. They were immediately exposed to colcemid (final concentration, $0.06 \mu g/mL$), rinsed twice in Hanks' balanced salt solution (HBSS), and exposed to hypotonic KCl (0.075 mol/L) for a total of 32 minutes at room temperature, including periods of mixing, standing, and centrifugation. They were then fixed twice in 3:1 methanol-acetic acid (vol/vol) for 15 minutes and ten minutes, after which slides were prepared by a flaming technique and natural aging for 0 to 7 days. Metaphase preparations

were G-banded by a modification of the trypsin method of Seabright.¹³

Chromosomal abnormalities were classified according to the International System of Human Cytogenetic Nomenclature (1985).¹⁴ The definition of an abnormal stem line was that proposed by the Second International Workshop on Chromosomes in Leukemia.¹⁵ A case was considered abnormal if it had an abnormal clone, regardless of the proportion of normal metaphases. For cases with more than one leukemic line due to clonal evolution, only the primary line was included in the analyses, with the exception of a single case of hypodiploidy in which the secondary line containing a translocation was used.

DNA content determination by flow cytometry. Leukemic marrow samples were stained with a DNA-specific dye, propidium iodide, and were analyzed by flow cytometry as previously described.¹⁶ The DNA index (ratio of DNA content in leukemic G_0/G_1 cells v normal diploid G_0/G_1 cells) was determined. This measure correlates closely with chromosome number (ploidy); hence leukemic cells with a normal chromosome number have a DNA index of 1.0.

Blast cell phenotyping. The diagnosis of ALL was based on morphological criteria of the French-American-British (FAB) group¹⁷ and negative myeloid-associated cytochemical findings. Cell-surface antigens were detected by a standard indirect immunofluorescence assay with monoclonal antibodies (MoAbs) to lym-

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phoid-associated antigens, including J5 (common ALL antigen, CALLA, CD-10), T11 (CD-2), T101 (CD-5), and T-3 (CD-3). Blast cells were also tested for surface and cytoplasmic immunoglobulin and rosette formation with sheep erythrocytes. Depending on the pattern of reactivity, the cells were classified as T, B, pre-B, CALLA⁺ early pre-B (common), or CALLA⁻ early pre-B (so-called undifferentiated), as previously described.¹⁸

Statistical analysis. Differences in the distribution of clinical and biological features among cases with hypodiploidy v other karyotypic subtypes were tested by the two-tailed Fisher exact test or the Pearson chi-square test. The Mann-Whitney test was used to examine the differences in the medians for continuous variables. To account for the problem of multiple significance testing,¹⁹ adjustment was made to the target significance level (0.05) by simply dividing it by the number of multiple tests.⁴ Hence a *P* value of at most 0.012 was required to declare statistical significance in those instances; the nominal *P* values are reported in Table 1.

Time-to-failure curves were constructed by the Kaplan-Meier procedure,²⁰ and differences were analyzed by the Cox-Mantel test,²¹ with adjustment for multiple comparisons.²² Time to failure was defined as the interval between achievement of remission and relapse or death due to any cause. Patients who did not enter complete remission were considered as failures and assigned a failure time of zero. A stratified log-rank test²³ was used to analyze differences in time to failure between the hypodiploid and hyperdiploid groups, after adjusting for age, translocation, leukocyte count, and serum lactic dehydrogenase level. The Cox proportional hazards model²⁴ was also used to determine if hypodiploidy added prognostic information beyond that of the above factors.

RESULTS

Thirty-one (7.6%) of the 409 patients had leukemic cell hypodiploidy (Table 2). These 14 boys and 17 girls ranged in age from 9 months to 17 years (median, 5 years). Of the 30 cases tested for blast cell immunophenotype, 17 had common ALL, 8 pre-B, and 3 T cell; the remaining two cases had blasts that expressed CALLA but were not tested for cytoplasmic immunoglobulin. The morphological subtypes of blast cells were FAB L1 in 26 patients and L2 in 5. Leukocyte counts ranged from 1.0 to $132 \times 10^9/L$ (median, $12.7 \times 10^9/L$), platelet counts from 3 to $483 \times 10^9/L$ (median, $53 \times 10^9/L$), and hemoglobin levels from 2.9 to 14.6 g/dL (median, 8.3 g/dL). Nine patients had massive

hepatosplenomegaly with a liver or spleen edge palpable more than 5 cm below the costal margins. Patients 2, 3, and 15 had initial CNS leukemia, and patient 24 had a mediastinal mass at diagnosis.

The modal chromosome numbers of the primary leukemic cell lines were 45 in 26 cases, 28 in two cases, and 26, 36, and 43 in one case each. Two or more leukemic lines derived from clonal evolution were found in seven patients (Nos. 3, 10, 11, 13, 16, 23, and 24). All but one case with near haploidy (26 chromosomes) had their blast cell G_0/G_1 DNA content determined by flow cytometry. Except for cases 10 and 23, each with 28 chromosomes (near haploidy), and case 22 with 36 chromosomes, the remaining 27 cases with 43 to 45 chromosomes had cellular DNA content values indistinguishable from normal diploid (DNA index = 1.0).

Hypodiploidy resulted from whole chromosome losses in 20 patients, from loss of a fragment of a chromosome involved in the translocation in 7, and from dicentric chromosome in 4. None of the cases showed a gain of whole chromosomes (Fig 1). Chromosome 20 was lost most frequently, and in three cases of common ALL a missing chromosome 20 was the only abnormality. Notably, none of the cases showed a loss of chromosome 21. Structural abnormalities included translocations in 21 cases (68%), deletions in 10 (32%), duplications in two, and an inversion in one. The structural abnormalities most frequently involved the short arms of chromosomes 9 and 12. Two nonrandom translocations were identified: t(1;19)(q23;p13.3) in two pre-B cases and t(9;12)(p11;p12) in three of common ALL. Deletions of chromosome 6 between q21 to q25 were found in four cases.

Table 1 shows the presenting features of patients according to chromosome ploidy. Hypodiploid cases had significantly lower leukocyte counts ($P = 0.001$) and lower serum lactic dehydrogenase levels ($P = 0.003$) than did those with a pseudodiploid karyotype but had a similar frequency of translocations. Hypodiploidy was associated with a higher frequency of leukemic cell chromosomal translocations ($P < 0.001$), higher serum lactic dehydrogenase levels ($P = 0.002$), and a greater proportion of patients with ages <2 or ≥ 10 years ($P = 0.015$) than was hyperdiploidy defined

Table 1. Distribution of Prognostic Factors by Ploidy Group

Variable	Feature	Hypodiploid (n = 31)	Pseudodiploid (n = 168)	Hyperdiploid >50 Chromosomes (n = 101)	Hyperdiploid 47-50 Chromosomes (n = 67)	Normal Diploid (n = 42)
Age (yr)	2 to 9	19	101	83	49	26
	<2 or ≥ 10	12	67 (0.90)*	18 (0.015)	18 (0.24)	16 (0.96)
Chromosomal translocation	Absent	10	55	85	45	42
	Present	21	113 (0.96)	16 (<0.001)	22 (0.001)	0 (<0.001)
Median leukocyte count ($\times 10^9/L$)	—	12.7	31.6 (0.001)	6.6 (0.17)	11.1 (0.61)	38.3 (0.017)
Median LDH level ($\times U/L$)	—	488	687 (0.003)	327 (0.002)	511 (0.32)	670 (0.07)

Abbreviations: NS, not significant; LDH, lactic dehydrogenase.

*Numbers in parentheses represent nominal significance values for comparisons of selected variables between hypodiploid cases and other ploidy groups.

Table 2. Clinical and Laboratory Data in 31 Cases of Hypodiploid ALL

Patient No.	Age (years)	Race	Immunophenotype	FAB Classification	Leukocyte Count ($\times 10^9/L$)	DNA Index	Karyotype	Duration of Remission (months)
1	0.8	W	Pre-B	L1	8.5	1.0	45,XY,-5,-9,+der(9),t(5;9)(q11;p21)	21
2	1	W	CALLA ⁺	L1	10.3	1.0	45,XY,-22,t(9;10)(q22;q11),t(10;11)(q24;q13)	39
3	2	W	Common	L2	39	1.0	45,XX,-20,t(2;16)(p13;p13)/45,XX,-20,-22,+mar,t(2;16)(p13;p13)	24
4	2	W	Common	L1	73	1.0	45,XX,-20	24+
5	2	W	Common	L1	116	1.0	45,X,-X,del(6)?(q21;q23)	13+
6	2	W	Pre-B	L1	18.2	1.0	45,XX,-4,-12,+der(12),t(4;12)(q21;p13)	11+
7	3	W	Common	L1	21.5	1.0	45,XX,robt(13;14)(q11;p11)	12
8	3	B	Common	L1	8.2	1.0	45,XY,-11,t(1;9)(q21;p21)	32
9	3	W	Common	L1	4.3	1.0	45,XY,-20	23+
10	4	W	Common	L2	1.0	0.6/1.18*	28,XY,-1,-2,-3,-4,-5,-6,-7,-9,-10,-11,-12,-13,-15,-16,-17,-19,-20,-22/56,XY,+X,+Y,+8,+8,+13,+14,+18,+18,+21,+21	49+
11	4	W	Common	L2	22.8	1.0	45,X,-Y/45,X,-Y,t(2;8)(p24;q12)	18
12	4	B	Common	L1	132	1.0	45,XY,del(X)(q26),-9,-12,+der(12),tdic(9;12)(p1?1;p1?2)	0
13	4	B	Pre-B	L1	9.8	1.0	45,XY,-8,-14,+der(14),t(7;8;14)(?q21.3;q32),del(5)(p14),del(17)(p12)/46,XY,-13,+der(13),t(13;?)(q34;?),del(17)(p12)	9+
14	5	W	Common	L1	44.6	1.0	45,X,-X,-9,+?der(9),t(9;?)(q34;?)	40+
15	5	W	Pre-B	L1	11.5	1.0	45,XX,-20,del(6)(q21)	26
16	5	W	Pre-B	L1	11.1	1.0	45,XY,-9,t(7;9)(cen;cen),dup(1)(?p33p35),del(8)(p22)/45,XY,-9,t(7;9)(cen;cen),dup(1)(?p33p35),del(8)(p22),-?E,+mar/46,XY,dup(1)(?p33p35)	27+
17	5	W	Pre-B	L1	2.1	1.0	45,XX,-12,-15,+tdic(12;15)(p11;p11),t(2;14)(?q24;q32)	10+
18	5	W	Common	L1	12.7	1.0	45,XY,-9,-12,+der(12),tdic(9;12)(p1?1;p1?2)	5+
19	6	W	Pre-B	L1	17.9	1.0	45,XX,-20,-19,+der(19),t(1;19)(q23;p13.3),del(8)(q22)	64+
20	7	W	Common	L1	22.8	1.0	45,XX,-8,-12,+der(12),t(8;12)(q?13;p13)	1+
21	8	W	Common	L1	4.0	1.0	45,X,-X,t(1;3)(q42;q28),del(6)(q21;q25),del(13)(q12;q14)	1+
22	10	W	Unknown	L1	5.9	0.76	36,XY,-2,-3,-6,-7,-10,-12,-13,-14,-16,-17,?dup(2)(q31;q37)	7+
23	11	W	T	L1	4.6	0.59/1.17*	28,XX,-1,-2,-3,-4,-5,-6,-7,-8,-9,-11,-12,-13,-15,-16,-17,-19,-20,-22/56,XX,+X,+X,+10,+10,+14,+14,+18,+18,+21,+21	37

Table 2. Clinical and Laboratory Data (Cont'd)

Patient No.	Age (years)	Race	Immunophenotype	FAB Classification	Leukocyte Count ($\times 10^9/L$)	DNA Index	Karyotype	Duration of Remission (months)
24	11	B	T	L2	1.7	1.0	43,XY,-1,+der(1),t(1;?) (?p36;?),-5,-6,+der(6), t(6;?)(?q27;?),-7,+der(7), t(7;?)(q36;?),-9,-10,-16, +der(16),t(16;?)(p13;?), t(9;13)(q11;p712)*	6
25	11	W	Common	L1	3.0	1.0	45,X,-X,t(1;3)(p34;p21), del(6)(q21q25)	11+
26	11	W	Common	L1	4.9	1.0	45,XX,-20	1+
27	13	W	Common	L1	32	1.0	45,XY,-22	10
28	14	W	T	L2	85.7	1.0	45,XX,-17,-12,+der(12), t(12;17)(p11;q11),inv(5)(p13 q14),del(10)(q22),del(11) (q22)	0
29	15	W	Pre-B	L1	34.6	1.0	45,XX,-13,-19,+der(19), t(1;19)(q23;p13.3),del(9) (p13)	57+
30	15	W	Common	L1	19.6	1.0	45,XY,-9,-12,+der(12), t(9;12)(p17;p172)	8+
31	17	W	CALLA ⁺	L1	34	Unknown	26,XX,-1,-2,-3,-4,-5, -6,-7,-8,-9,-10,-11, -12,-13,-15,-16,-17, -18,-19,-20,-22	6

Abbreviations: FAB, French-American-British; W, white; B, black; CALLA, common acute lymphoblastic leukemia antigen.

*Values for primary and secondary leukemic lines.

+Contained three other similar leukemic lines, one with an additional translocation, from clonal evolution.

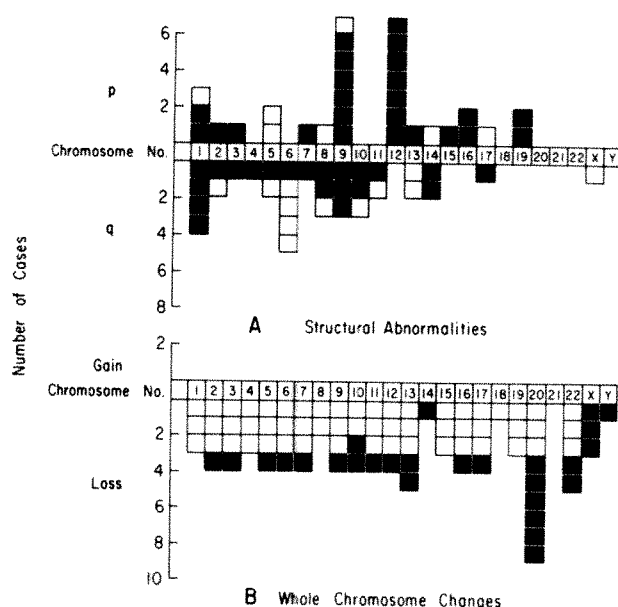


Fig 1. Structural and numerical chromosomal abnormalities in hypodiploid cases. (A) The closed blocks represent specific chromosomes involved in translocations; open blocks represent all other abnormalities not associated with translocations. Each breakpoint is depicted once. The structural abnormalities most frequently involved the 9p and 12p arms. (B) The open blocks represent chromosomal losses in cases of near haploidy; closed blocks represent losses in other hypodiploid cases. The loss of chromosomes most frequently involved number 20 and did not involve number 21.

by >50 chromosomes. The frequency of translocations was also significantly higher in hypodiploid cases compared to those with 47 to 50 chromosomes ($P = 0.001$). There were no significant differences between the hypodiploid group and others in sex ratio, race distribution, frequency of CNS leukemia or mediastinal mass, liver or spleen size, hemoglobin level, platelet count, and distribution of FAB morphological subtypes and immunophenotypes (data not shown).

Forty-two cases in this study, half with a T cell phenotype, had only normal chromosomes on marrow karyotype analyses. As expected, comparison of this ploidy group with hypodiploid cases disclosed significant differences in frequency of translocations and immunophenotypes; however, with improved cytogenetic techniques the finding of "normal" diploid karyotypes is becoming increasingly rare in our laboratory.²⁵ Hence the normal karyotypes most likely reflect normal residual marrow elements in cases in which the leukemic metaphase cannot be easily studied (eg, T cell ALL).

Since the distribution of ploidy groups was similar in the three successive clinical trials, patients were combined for analysis of time to failure. Patients with hypodiploid karyotypes were more likely to fail therapy than were those with >50 chromosomes ($P = 0.007$, Fig 2). There was no significant difference in outcome between the hypodiploid and the other ploidy groups.

Using the stratified log-rank test, we could find no difference in time to failure between the hypodiploid cases and hyperdiploid >50 group after adjustment for the selected

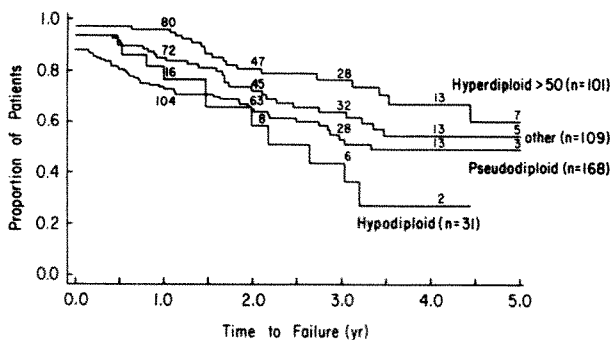


Fig 2. Kaplan-Meier estimates of time-to-failure rates. Patients with leukemic cell hypodiploidy fared significantly worse than those with chromosomes >50 ($P = 0.007$). There was no significant difference in the failure rates between hypodiploid cases and those with pseudodiploidy ($P = 0.99$) or those with other ploidy, defined as 47 to 50 chromosomes or normal karyotype ($P = 0.33$). Numbers along the curves represent patients at risk of failure at various times.

important prognostic factors ($P = 0.43$). Cox regression analysis showed that hypodiploidy added only marginal prognostic information beyond that conferred by leukocyte count, age, serum lactic dehydrogenase level, and chromosomal translocation ($P = 0.08$).

DISCUSSION

In this large series of pediatric ALL cases with completely banded karyotypes, we have shown that hypodiploidy occurs infrequently (7.6% of cases) but is clearly associated with an adverse treatment outcome. In contrast to cases with >50 chromosomes, hypodiploid cases were more likely to have ages <2 or ≥ 10 years, higher serum lactic dehydrogenase levels, and leukemic cell chromosomal translocations. Age has consistently been an independent predictor of treatment outcome in childhood ALL, with patients <2 years or ≥ 10 years faring much worse than children in the intermediate age group.²⁶ Recently, we reported that increased serum lactic dehydrogenase levels confer a poor prognosis and may provide a more reliable estimate of the total body burden of leukemic cells than does leukocyte count alone.²⁷ There is now compelling evidence that leukemic cell chromosomal translocations are the karyotypic abnormality with the most profound impact on treatment outcome.^{4,8,28} In our experience patients with any chromosomal translocation have a sixfold greater risk of early treatment failure than do those without such abnormalities.²⁸ Thus the poor treatment responsiveness of hypodiploid ALL can be attributed, at least in part, to its associated high-risk clinical and biological features. The small number of hypodiploid cases precluded further subset analyses of risk features among this group.

We think it noteworthy that patients in the hypodiploid and pseudodiploid groups showed equally poor responses, despite lower leukocyte counts, lower serum lactic dehydrogenase levels, and a lack of cytogenetic abnormalities with known adverse influence, such as $t(4;11)$, $t(8;14)$, and $t(9;22)$ in the former group. Since ages and frequencies of chromosomal translocations were similar in these subsets of

patients, one could postulate additional factors underlying the poor outcome in hypodiploid cases. In this respect it is tempting to speculate on the significance of chromosome loss in relation to the concept of "anti-oncogenes"^{29,30} or recessive mutations in somatic cells,^{31,32} as proposed for certain childhood malignancies. This theory holds that dysfunction or loss of anti-oncogenes may be important in tumorigenesis, by allowing abnormal expression of proto-oncogenes or the loss of differentiation products that are encoded by anti-oncogenes.^{29,30} Alternatively, the malignancy may arise as a consequence of recessive mutations in somatic cells that are unmasked by genetic events that resulted in the loss of the unaffected gene in the homologous chromosome.^{31,32} In any event, hypodiploid cells may be more likely than other cells to lose their suppressor genes or to express recessive oncogenes, possibly leading to more aggressive disease.

Near-haploid ALL is rare. The reported cases are associated with 26 to 28 chromosomes in leukemic cells that retain at least one homolog of each chromosome, some being disomic. This type of leukemia may have a second line containing twice the near-haploid number, as seen in two of the three cases in this study. This doubling of chromosome number is a form of clonal evolution. In general these patients have a very poor outcome.³³ Two of the three patients in this study with near haploidy have failed.

Two types of nonrandom translocations were observed: the $t(1;19)(q23;p13.3)$ in two cases of pre-B ALL^{18,34,35} and the $tdic(9;12)(p1?1;p1?2)$ in three cases of common ALL. The $tdic(9;12)$ has recently been described in association with ALL of B cell precursors, hypodiploidy, and male predilection.³⁶ It may be important that the *c-Kirsten-ras* 2 proto-oncogene is located at 12p12,³⁷ the region involved in this translocation.

The finding of multiple leukemic lines at diagnosis is not unusual in ALL. Kaneko et al³⁸ reported this result in about one fifth of 50 consecutive cases of ALL (31 in children and 19 in adults). At our center 26% of 116 consecutive cases had multiple lines at the time of diagnosis.³⁹ In most of them the multiple lines were the result of clonal evolution; only rarely did they appear to represent two independent stem lines (ie, biclonal).³⁹ All seven cases of multiple stem lines that we observed had evidence of clonal evolution. The clinical significance of ALL defined by two or more leukemic lines remains unknown.

Studies comparing cellular DNA content measurements by flow cytometry with cytogenetic determinations of leukemic cell ploidy have disclosed an excellent correlation for both adults and children.^{40,41} The advantages of flow cytometry are not simply that the measurements are relatively easy and rapid to perform, but also that a high degree of statistical accuracy is obtained through analysis of large numbers of cells independent of low proliferative rate and mitotic index. However, this method cannot distinguish pseudodiploid cells with translocations or other abnormalities from normal cells and does not reveal the identity of individual chromosomes gained or lost. Moreover, it is not sensitive enough to detect a small gain or loss of total chromosomal DNA. All of the 27 cases with 43 to 45 chromosomes that were tested in this study had a DNA index value of 1.0 (see Table 2) and hence

could not be identified as hypodiploid with use of this technique.

Recently, cytogenetic techniques have improved to the degree that virtually all cases of childhood ALL can be shown to have clonal chromosome abnormalities.²⁵ While the distinction between hypodiploid 43-45 and pseudodiploid ALL cases may not be critical for treatment planning, as both groups have poor outcomes, cytogenetic analysis identifies the subgroup with translocations and therefore a worse

prognosis and could contribute to the understanding of leukemogenesis by discovery of nonrandom changes. The basis for the nonrandom nature of chromosomal loss in childhood ALL and the associated poor treatment outcome remains to be elucidated.

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Effects of B Cell Stimulatory Factor-1/Interleukin 4 on Hematopoietic Progenitor Cells

By Christian Peschel, William E. Paul, Junichi Ohara, and Ira Green

B cell stimulatory factor-1 (BSF-1)/Interleukin 4 (IL 4) is a T cell product originally characterized on the basis of its actions on B lymphocytes. Recently it has been reported that BSF-1 activates T cell and mast cell lines. We now provide evidence that BSF-1, purified to homogeneity, also has a broad spectrum of activity on hematopoietic progenitor cells (HPC). However, like its action on B cells, proliferative effects were only observed when BSF-1 was combined with an additional factor. Thus BSF-1, in costimulation with recombinant G-CSF, enhances the proliferation of granulocyte-macrophage progenitor cells (CFU-GM). BSF-1 increases the proliferation of CFU-e in the presence of recombinant erythropoietin (rEPO). Furthermore, BSF-1 induces, together with rEPO, colony formation by primitive

erythroid (BFU-e) and multipotent (CFU-mix) progenitor cells comparable to that observed with rEPO and interleukin 3 (IL 3). BSF-1 is also active as a megakaryocyte colony-stimulating factor; in combination with recombinant interleukin 1, rEPO or the supernatant of the T cell hybridoma FS7-20.6.18, BSF-1 induces megakaryocyte colony formation (CFU-Mk). The same factors that synergize with BSF-1 also enhance CFU-Mk proliferation induced by IL 3. Although the precise mechanisms of action of BSF-1 on HPC is not yet known, we propose that BSF-1 represents an activation factor for HPC and prepares the progenitor cells to respond to specific growth or differentiation factors.

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B CELL stimulatory factor-1 (BSF-1)/interleukin 4 is an ~20,000 mol wt T cell product that was originally described as a costimulant with anti-IgM antibodies for the entry of resting B cells into the S phase of the cell cycle.¹⁻³ It is now known that BSF-1 increases the expression of class II MHC molecules on resting B cells,^{4,5} promotes secretion by B lymphocytes of IgG₁ and IgE,^{6,7} and induces proliferation of colony-forming B cells (C. Peschel, manuscript in preparation). In addition to these multiple actions on B lymphocytes, BSF-1 has recently been shown to promote the growth of helper T cell lines and to be identical with a factor that enhances the growth of mast cell lines in response to interleukin 3 (IL 3).⁸⁻¹¹ In this study we now demonstrate that BSF-1 has activities on several hematopoietic progenitor cell types.

The proliferation and differentiation of hematopoietic progenitor cells (HPC) in vitro is regulated by a family of specific growth factors, usually referred to as colony stimulating factors (CSFs).¹² Interleukin 3 (IL 3), also designated as multi-CSF, promotes the in vitro proliferation of murine pluripotent hemopoietic stem cells, primitive erythroid (BFU-e), megakaryocytic (CFU-Mk), and granulocyte-macrophage (CFU-GM) progenitor cells and mast cells.¹³⁻¹⁶ The effects of other growth factors are mainly restricted to one cell lineage. A group of CSFs regulates the growth of CFU-GM. G-CSF and M-CSF preferentially stimulate the proliferation of granulocytes and macrophages respectively,

whereas GM-CSF stimulates the production of both granulocytic and macrophage cells.^{17,18} In recent experiments it became evident that recombinant GM-CSF has a broader spectrum of activity and provides stimulating activity for erythroid and multipotent progenitor cells^{19,20} and megakaryocytic progenitor cells.¹⁶ Erythropoietin (EPO) has similar properties as a growth factor for mature erythroid progenitor cells (CFU-e)²¹ and in combination with an additional activity²² mainly provided by IL 3¹⁵ for BFU-e. The effect of erythropoietin on CFU-e and BFU-e can also be enhanced by another factor designated as erythroid potentiating activity (EPA).^{23,24} The in vitro proliferation of murine CFU-Mk has been proposed to be regulated by two factors.²⁵ CSF-Mk is identical with IL 3²⁶; a megakaryocyte potentiating activity (MkPA) that enhances the growth of megakaryocyte colonies in the presence of IL 3 has been reported to be present in several crude cell supernatants.^{16,26,27}

The proliferative activity of the CSFs is restricted to hematopoietic cells, with the exception that IL 3 also has an effect on the differentiation of immature T cells²⁸ and stimulates the proliferation of B cell precursors.²⁹ By contrast, BSF-1, which has major actions on mature B and T cells, is shown here to have significant action on hematopoietic progenitor cells. In costimulation with different growth factors, BSF-1 induces in vitro proliferation of multipotent (CFU-mix) and restricted myeloid (CFU-GM), erythroid (BFU-e, CFU-e), and megakaryocyte (CFU-Mk) progenitor cells.

MATERIALS AND METHODS

Animals. Female Balb/c mice, 6 to 10 weeks of age, were obtained from the breeding facilities of the National Institutes of Health, Bethesda, MD, and utilized throughout these studies.

Cell separation. Bone marrow cells were obtained from femurs and tibiae, and single cell suspensions were prepared in McCoy's 5A medium (Flow Laboratories, McLean, VA) containing 5% fetal calf serum (FCS; Hyclone, Logan, UT), by passage through a thin needle. The McCoy's 5A medium was supplemented with 1 mmol/L sodium pyruvate, MEM Vitamins (1:100), MEM nonessential (1:100), and essential (1:50) amino acids, 2 mmol/L L-glutamine, 16 µg/mL L-asparagine, 10 µg/mL L-serine, 10 mmol/L HEPES buffer, penicillin-streptomycin, and 5×10^{-5} mmol/L 2-mercapto-

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ethanol, and adjusted to pH of 7.2. After enrichment of the mononuclear cell fraction (MNC) by centrifugation over Ficoll-Hypaque (FH, density 1.077g/mL, Pharmacia, Piscataway, NJ), interface cells were depleted of adherent cells by two consecutive adherence procedures in tissue culture dishes (100-mm diameter, Falcon, MA) at a cell density of 1×10^6 MNC/mL. Nonadherent cells obtained after the second incubation period of 90 minutes were incubated with a cocktail of appropriate dilutions of Thy1.2, Lyt-1, and Lyt-2 antibodies to remove T cells (Beckton Dickinson, Sunnyvale, CA) for 30 minutes on ice; after centrifugation cells were suspended in a 1:10 solution of low-tox-M rabbit complement (Accurate Chemical, Westbury, NY) with a monoclonal mouse antirat κ chain antibody (MAR 18.5) and incubated for 45 minutes at 37°C. This adherent cell and T cell-depleted bone marrow cell population was used in all experiments. In certain experiments a monoclonal antibody (MoAb) produced by the hybridoma cell line RA33A16.1 (obtained from American Type Culture Collection, Rockville, MD), which recognizes the B220 antigen, was additionally used with complement to remove B and pre-B cells.³⁰

Growth factors. BSF-1 was purified from supernatant fluids of 4 β -phorbol-12 β -myristate-12 α -acetate (PMA) activated EL-4 cells by a combination of affinity chromatography and high-performance liquid chromatography (HPLC). Briefly, 4L of EL-4 Sn was passed over an affinity column of 10 mL Sepharose-4B conjugated with 20 mg γ -globulin from a monoclonal anti-BSF-1 (11B11) ascites³¹ at a flow rate of 4 mL/min. After washing the column thoroughly with 2 L glass-distilled water, BSF-1 was eluted with 0.1% trifluoroacetic acid (TFA). The eluate was loaded onto a C18-reversed phased HPLC column (Supelco, Inc, Bellefonte, PA) with a Perkin-Elmer HPLC system (Perkin-Elmer Corp, Norwalk, CT) as described previously.³² The column was developed with a linear gradient of 35% to 55% acetonitrile in 0.1% TFA over 60 minutes at a flow rate of 0.5 mL/min. The detailed procedure will be described elsewhere (J. Ohara et al, manuscript in preparation). The resultant BSF-1 migrated as a single molecular species of mol wt 20,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The N-terminal amino acid sequence of this material is identical to that inferred from the nucleotide sequences of cDNA clones of BSF-1.^{33,34} A U/mL of BSF-1 is the concentration required for half maximal stimulation of ³H-thymidine uptake by purified splenic B cells (10^5 cells/mL) stimulated with goat antimouse IgM antibody (5 to 10 μ g/mL).³²

Highly purified IL 3 was kindly provided by J. N. Ihle, Frederick, MD.³⁵ Recombinant human erythropoietin (rEPO) purified by sequential chromatography was purchased from Amgen (Thousand Oaks, CA). Recombinant human interleukin 1 α (IL 1) was generously provided by Hoffman LaRoche (Nutley, NJ), and recombinant human G-CSF was kindly provided by Amgen.³⁶ The supernatant of the T cell hybridoma FS7-20.6.18 that has recently been described to contain megakaryocyte potentiating activity²⁷ was kindly supplied by P. Marrack (National Jewish Hospital, Denver, CO).

Hematopoietic progenitor assays. Colony-forming assays were performed in a modified agar culture system as previously described.³⁷⁻³⁹ Nonadherent T cell-depleted bone marrow cells were suspended in culture medium containing 10% heat-inactivated FCS and 0.3% Bacto-Agar held at 37°C (Difco, Detroit). Aliquots of 0.25 mL of this mixture containing 2×10^4 or 4×10^4 viable cells were pipetted into Nunclon 4-well multidishes (Nunc, Denmark). After solidification at room temperature, a liquid overlayer of 0.25 mL culture medium containing 10% fetal calf serum (FCS) and the growth factors to be studied was added. The growth factors tested in the different progenitor cell assays are shown in Table 1. All stimulating factors suspended in the liquid overlayer were applied in double concentration to achieve final dilutions in the whole culture

system as described in Table 1 and Results. After incubation in 6% CO₂ in fully humidified atmosphere for 2.5 to seven days, the whole agar cultures were fixed with 2.5% glutaraldehyde, washed in distilled water for four hours, mounted on microscopic slides, and dried on a slide warmer. May-Grünwald-Giemsa,³⁷ benzidine,⁴⁰ and acetylcholinesterase⁴¹ stainings were performed on the dried agar sheets as described; the slides were permanently mounted with Permount (Fisher, Fairlawn, NJ); and numbers of colonies or clusters were scored under a light microscope.

A summary of the progenitor cell assays, definition of colony types, and factors used, as detailed below, are also given in Table 1.

CFU-GM assay. 2×10^4 cells/well were incubated with G-CSF 10 to 200 U/mL alone or in combination with BSF-1 30 U/mL or IL 1 5 U/mL or IL 3 50 U/mL for six to seven days. Granulocytic clusters (10 to 50 cells) and colonies (>50 cells) were distinguished in cultures stimulated with G-CSF $\pm$ BSF-1. In IL 3-containing cultures only colonies were counted.

CFU-e assay. 2×10^4 cells/well were stimulated with rEPO 0.1 U/mL or 1.0 U/mL $\pm$ BSF-1 30 U/mL or BSF-1 30 U/mL alone. The CFU-e number was evaluated at 2.5 to three days of incubation in preparations stained for benzidine to identify hemoglobin-containing cells. CFU-e were defined as clusters of more than eight normoblast-like hemoglobinized cells. Such clusters do not contain more than 20 normoblasts.

BFU-e and CFU-Mix assay. For assays of erythroid (BFU-e) and mixed erythroid (CFU-mix) progenitor cells, 4×10^4 cells/well were incubated with rEPO 2.0 U/mL in combination with IL 3 50 U/mL or BSF-1 30 U/mL for seven days and stained with benzidine. Such colonies generally contain more than 50 cells. Control cultures containing medium alone, IL 3, or BSF-1 alone were always included. The composition of mixed erythroid colonies was also evaluated in preparations stained for acetylcholinesterase to identify megakaryocytes. In these preparations erythroid cells were identified by their typical morphology. Mixed erythroid colonies that contained erythroid cells and megakaryocytes and/or granulocytes were distinguished from pure erythroid colonies.

CFU-Mk assay. 4×10^4 cells/well were stimulated with BSF-1 30 U/mL, IL 3 1 to 100 U/mL alone or in combination with IL 1 5 U/mL, rEPO 2.0 U/mL or FS7-20.6.18-conditioned medium (CM) 5% as source of MkPA. Colonies were defined as aggregates of more than three large acetylcholinesterase-positive cells⁴¹ after seven to eight days of incubation. In cultures stimulated with EPO and IL 3 or BSF-1, mixed colonies containing megakaryocytes, erythroid cells, and/or granulocytic/monocytic cells were distinguished from pure megakaryocytic colonies.

Use of anti-BSF-1 antibody. In all hematopoietic progenitor cell assays described above, the specificity of the biological activity induced by added BSF-1 was confirmed by the addition of a monoclonal rat anti-BSF-1 antibody, designated as 11B11³¹ to the cultures. The anti-BSF-1 antibody was used in dilutions of 1:500 of ascitic fluid in selected cultures and was added to the cultures together with BSF-1 or other growth factors during the whole incubation period.

Statistical analysis. All cultures on a single day were performed at least in duplicate, and colony counts of parallel cultures were pooled. The same experiments were repeated three to four times over a period of time, and the results were expressed as mean values of these different experiments. The differences between corresponding experiments have been examined by the two-tailed Dunnet test.

RESULTS

The purpose of this study was to determine the effect of BSF-1, alone or together with other CSFs, on the growth of hematopoietic progenitor cells. Using adherent cell-depleted

Table 1. Hematopoietic Progenitor Cell Assays, Colony Types, and Factors Used

Hematopoietic Progenitor Cell Type	Definition of In Vitro Colonies	Factors Used* for Stimulation	Staining Procedures
CFU-GM: Restricted myelomonocyte progenitor cell	Clusters (10-50 cells) or colonies (>50 cells) of granulocytes and/or macrophages, scored after 7 days of incubation	BSF-1 30 U/mL G-CSF 10-200 U/mL IL 3 50 U/mL IL 1 5 U/mL	May-Grünwald-Giemsa
CFU-e: Restricted erythroid progenitor cell	Hemoglobin-containing clusters of more than 8 cells, scored after 2.5-3 days of incubation	EPO 0.1-1.0 U/mL BSF-1 30 U/mL	Benzidine May-Grünwald-Giemsa
BFU-e: Restricted primitive erythroid progenitor cell	Hemoglobin-containing single or multiple colonies of more than 50 cells, scored after 7 days of incubation	EPO 2.0 U/mL BSF-1 30 U/mL IL 3 50 U/mL	Benzidine May-Grünwald-Giemsa
CFU-mix: Multipotent hemopoietic progenitor cell	Colonies consisting of erythroid and megakaryocytic and/or myelomonocytic cells, scored after 7 days of incubation	EPO 2.0 U/mL BSF-1 30 U/mL IL 3 50 U/mL	Benzidine Acetylcholinesterase May-Grünwald-Giemsa
CFU-MK: Restricted megakaryocytic progenitor cell	Colonies of more than 3 megakaryocytes, scored after 7-8 days of incubation	IL 3 1-100 U/mL BSF-1 30 U/mL EPO 2.0 U/mL IL 1 5 U/mL FS7-20.16.8 CM 5%	Acetylcholinesterase

*The factors listed were used alone or in combination with each other, as described in results.

and T cell-depleted bone marrow cells in a modified agar culture technique,³⁷⁻³⁹ no significant cell proliferation was observed without growth factors after seven to 14 days of incubation. Therefore, effects of constituent production of growth factors in situ could be neglected in our culture system. Stimulation with IL 3, EPO, or G-CSF led to a proliferation of myelomonocytic and megakaryocytic, erythroid and granulocytic colonies respectively, as had been previously described for these factors.¹² In cultures stimulated with BSF-1 alone in concentrations of 30 to 1,000 U/mL, only a few small granulocyte/macrophage cell clusters and a few groups of two to three megakaryocytes developed. The effects of BSF-1 in combination with other growth factors is now described in the following sections.

Proliferation of granulocyte-macrophage progenitor cells (CFU-GM). First, to determine the maximal number of granulocyte/macrophage colonies that could be observed, IL 3, a powerful multi-CSF, was used as a growth factor. Stimulation with IL 3 (50 U/mL) induced a mean of 272 large colonies consisting of granulocytes and/or macrophages/20,000 cells plated in three different experiments. As described above, BSF-1 alone did not stimulate the proliferation of myeloid colonies; only four to seven granulocytic clusters of more than ten cells developed in these experiments.

We then tested the effects of BSF-1 together with G-CSF. After stimulation for seven days with various concentrations of G-CSF alone, a dose-dependent increase in the number of clusters (10 to 50 cells, Fig 1A) and of colonies (>50 cells, Fig 1B) consisting of mature granulocytes was observed. The addition of 30 U/mL of BSF-1 to various concentrations of G-CSF led to a significant increase in size and number of granulocytic clusters and colonies (Figs 1A and 1B). The use of higher concentrations of BSF-1 did not further increase the number of colonies derived from CFU-GM (Fig 2).

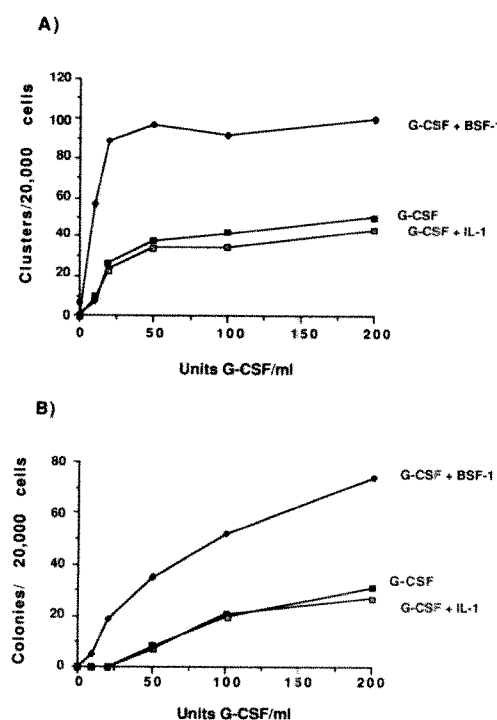


Fig 1. Role of BSF-1 in the stimulation of CFU-GM. Cultures were stimulated with various amounts of G-CSF alone or in combination with BSF-1 (30 U/mL) or IL 1 (5 U/mL). Clusters of 10 to 50 cells (A) and colonies of more than 50 cells (B) were counted after six days of incubation. Values represent the mean of three different experiments.

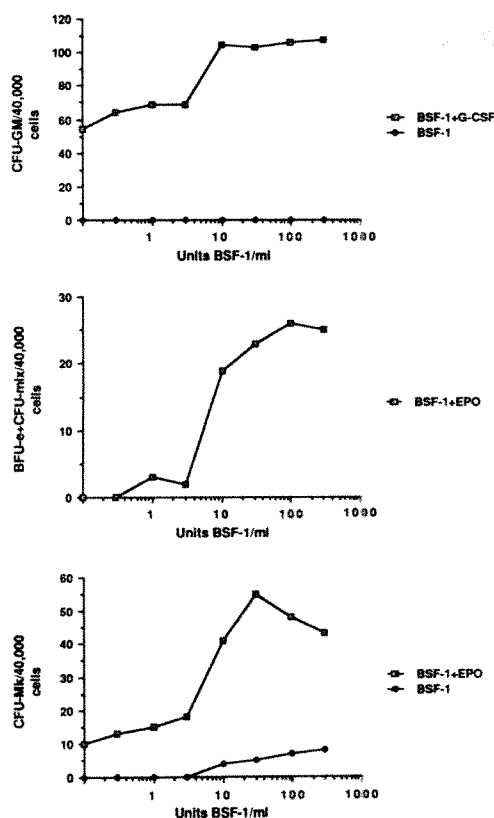


Fig 2. Effect of different concentrations of BSF-1 on proliferation of hematopoietic progenitor cells. Cultures were stimulated with various amounts of BSF-1 alone or in combination with G-CSF 200 U/mL (CFU-GM) or EPO 2.0 U/mL (BFU-e, CFU-mix, CFU-Mk). Colonies were counted after six days (CFU-GM) or seven days (BFU-e, CFU-mix, CFU-Mk) of incubation. Values represent the mean of triplicate cultures of one typical experiment.

Morphologically, most of these colonies consist of less differentiated granulocytic cells as compared to cells in colonies stimulated with G-CSF alone. In addition, some colonies with monocyte-macrophage features appeared. The addition of IL 1 to G-CSF had no enhancing effect on granulocytic colony formation (Figs 1A and 1B).

When cultures were stimulated with G-CSF and BSF-1 and incubated for 14 days, we observed development of macrophage colonies, whereas in control cultures containing medium alone, G-CSF or BSF-1 alone, no colony formation of any kind could be detected at this time (data not shown). The granulocytic colonies that had been present at seven days of culture have by 14 days degenerated and are no longer visible. The appearance of macrophage colonies at 14 days suggests that BSF-1 in the presence of G-CSF also can induce a commitment and proliferation of CFU-GM along the monocyte-macrophage cell lineage.

Monoclonal anti-BSF-1 antibody blocked the enhancing effect of BSF-1 on the formation of granulocytic colonies. In such cultures the number of colonies was reduced to values obtained with G-CSF alone (Table 2). This result indicates that the active principal in the BSF-1 preparations is, indeed, BSF-1.

Proliferation of mature erythroid progenitor cells (CFU-e).

CFU-e were stimulated with optimal (1.0 U/mL) or suboptimal (0.1 U/mL) amounts of recombinant erythropoietin (rEPO), as previously determined in titration experiments (data not shown), and numbers of erythroid clusters were evaluated in benzidine-stained preparations at 2.5 to three days of incubation (Fig 3). A few cell clusters consisting of nonhemoglobinized basophilic normoblasts developed in the absence of exogenously added EPO, most probably under the influence of the small amounts of EPO that are present in FCS. BSF-1 significantly enhanced the effect of 0.1 U/mL and 1.0 U/mL of EPO on CFU-e proliferation induced; the background proliferation of CFU-e without exogenous EPO was also increased by BSF-1 (Fig 3).

In cultures with BSF-1 alone or with 0.1 U/mL EPO $\pm$ BSF-1, only nonhemoglobinized basophilic normoblasts were observed, whereas in the presence of 1.0 U/mL EPO, BSF-1 enhanced the proliferation of fully hemoglobinized erythroid clusters. These data demonstrate that BSF-1 has an enhancing effect on the in vitro proliferation of mature erythroid progenitor cells and resembles the effects described for EPA.²³ As shown in Table 2, the enhancing activity of BSF-1 on CFU-e was abrogated by anti-BSF-1 antibody.

Proliferation of primitive erythroid (BFU-e) and multipotent (CFU-mix) progenitor cells. In contrast to CFU-e, BFU-e are evaluated by colony formation at seven days of culture, at which time clusters arising from CFU-e have degenerated. Under our culture conditions rEPO alone had almost no colony-stimulating activity on BFU-e (Fig 4). In cultures containing IL 3 alone, no hemoglobin-containing erythroid cells or morphologically recognizable normoblasts could be detected. Only pure granulocyte-macrophage and megakaryocyte colonies and colonies consisting of both of these cell types were observed. Thus a commitment to the erythroid cell line was not observed as a result of stimulation by IL 3 alone (data not shown). However, combination of EPO with IL 3 led to the proliferation of pure erythroid colonies and of mixed erythroid colonies (Fig 4). When BSF-1 was combined with EPO, a similar effect on BFU-e and CFU-mix was observed; both pure erythroid and mixed erythroid colonies were induced. Thus IL 3 and BSF-1 had comparable activities in allowing proliferation of BFU-e and of multipotent progenitor cells in the presence of EPO, as assessed in four different experiments (Fig 4). The effect of BSF-1 on BFU-e and CFU-mix was clearly detectable with BSF-1 10 U/mL and was not significantly enhanced with higher concentrations (Fig 2).

The mixed colonies, induced by EPO together with IL 3 or BSF-1, consisted of erythroid cells, megakaryocytes, and not clearly identifiable blast-like mononuclear cells. In the presence of IL 3, mixed colonies also contained granulocytes and macrophages, whereas BSF-1 together with EPO did not induce significant differentiation to the granulocytic cell lineage. In experiments in which the effect of the anti-BSF-1 antibody was examined, the effect of BSF-1 on BFU-e and CFU-mix was completely blocked by 11B11 (Table 2).

Proliferation of megakaryocyte progenitor cells (CFU-Mk). When bone marrow cells were incubated with BSF-1 alone, a few groups of three to four megakaryocytes and an increased number of single megakaryocytes could be identi-

Table 2. Effect of Anti-BSF-1 Antibody on the Number of Colonies Arising from Proliferation of Hematopoietic Precursor Cells

CFU-GM			CFU-e			BFU-e and CFU-mix†			CFU-Mk			CFU-Mk		
11B11			11B11			11B11			11B11			11B11		
Growth Factor	-	+	Growth Factor	-	+	Growth Factor	-	+	Growth Factor	-	+	Growth Factor	-	+
BSF-1	3/0*	2/0	Medium	4	3	Medium	0	0	Medium	0.5	0	BSF-1 + IL 1	57	0
G-CSF 50	20/6	ND	rEPO 1.0	55	ND	rEPO	3	ND	rEPO	9	ND	BSF-1 + T-CM‡	43	85
G-CSF 50 + BSF-1	53/34	30/9	BSF-1	9	2	BSF-1	0	0	T-CM‡	5	ND	IL 3	87	85
G-CSF 100	30/12	ND	BSF-1 + rEPO 1.0	89	52	rEPO + BSF-1	22	2	BSF-1	18	0	IL 3 + T-CM‡	152	144
G-CSF 100 + BSF-1	53/43	23/12				rEPO + IL 3	31	28	BSF-1 + rEPO	45	12	IL 3 + rEPO	160	165

*The first value represents the number of clusters of 10-50 cells, the second value represents the number of colonies of >50 cells.

†Pure and mixed erythroid colonies were counted together in this experiment.

‡T-CM = FS7-20.6.18 CM 5%.

fied by their positive staining for acetylcholinesterase (Fig 5A). IL 3 stimulates a substantial number of megakaryocyte colonies (Fig 5B). Recently a megakaryocyte-potentiating activity has been described that enhances megakaryocyte colony formation induced by IL 3.²⁵ Therefore several growth factors were tested for a megakaryocyte potentiating activity (Mk-PA) in costimulation with BSF-1 and with IL 3. IL 1, EPO, and the supernatant of the T cell hybridoma FS7-20.6.18, which has recently been shown to be a source of Mk-PA,²⁷ all significantly increased the number of megakaryocytic colonies obtained with BSF-1 (Fig 5A). This megakaryocyte-potentiating activity was also reflected in an increased cell number of the individual megakaryocyte colonies. IL 1 alone had no influence on megakaryocyte proliferation, whereas FS7-20.6.18-conditioned medium and EPO induced a small number of megakaryocyte colonies comparable to BSF-1. In cultures stimulated with BSF-1 and EPO, 14 of 63 megakaryocyte colonies shown in Fig 5A also contained erythrocytes. IL 1, FS7-20.6.18-CM, and EPO also significantly ($P < 0.05$) increased the number and size of megakaryocyte colonies obtained with IL 3 (10 U/mL or 100 U/mL). In the presence of EPO and IL 3, 25 of 139 colonies shown in Fig 5B contained both erythroid cells and megakaryocytes. These data demonstrate that the same potentiating factors have synergistic activities with both BSF-1 and IL 3, although at a different quantitative level. This suggests a Mk-CSF-like activity for BSF-1. The stimulating potency of BSF-1 on CFU-Mk was similar to its effects on CFU-GM and BFU-e (Fig 2). To determine whether BSF-1 also has Mk potentiating activity, we tested the capacity of BSF-1 to enhance the number of megakaryo-

cyte colonies that appear in response to IL 3 (Table 3). Even at suboptimal concentrations of IL 3, BSF-1 had no additive or synergistic effect on CFU-Mk proliferation, suggesting that BSF-1 lack Mk potentiating activity.

Anti-BSF-1 antibody abrogated the activity of BSF-1 on CFU-Mk, whereas the antibody had no influence on megakaryocyte colony formation induced by IL 3 (Table 2). The anti-BSF-1 antibody has also been used to further characterize the activity of FS7-20.6.18 CM. This supernatant has been shown to play a role in Ia induction on CFU-Mk,²⁷ analogous to an activity that is known for BSF-1 on B lymphocytes.^{4,5} Therefore to determine whether the activity of FS7-20.6.18 CM was due to BSF-1, we added anti-BSF-1 antibody. No blocking of the enhancement of CFU-Mk proliferation by FS7-20.6.18 CM in costimulation with IL 3 was observed, thus demonstrating that the MkPA of this T cell supernatant is not identical with BSF-1.

Depletion of B cells has no influence on the effect of BSF-1 on hematopoietic progenitor cells. Hematopoietic growth factors can exert their specific activity directly on the progenitor cell, or they can induce other cells like macrophages or T lymphocytes to release other growth factors that activate the particular target cell.⁴² To avoid these indirect effects, adherent cells and T lymphocytes were depleted in all experiments prior to the agar culture. Recently it has been described that activated B cells also can produce cytokines that affect in vitro stem cell proliferation.⁴³ Since BSF-1 is a known B cell activation factor, we attempted to exclude the possibility that the activity of BSF-1 on hematopoietic progenitor cells might be caused by an indirect effect on B lymphocytes in our bone marrow population. For this purpose we additionally depleted bone marrow cells of pre-B and B cells by incubation with anti-B220 antibody (RA33A16.1) and complement. The effects of BSF-1 and other growth factors on hematopoietic progenitor cells was tested on bone marrow cells depleted of B cells, adherent cells, and T cells and compared with cells depleted only of adherent cells and T cells. As shown in one typical experiment in Table 4, the number of colonies per culture increased after depletion of B220⁺ cells, probably due to an enrichment of hematopoietic progenitor cells. The activity of BSF-1 on all progenitor cells tested was not affected by the depletion of B and pre-B cells from the bone marrow.

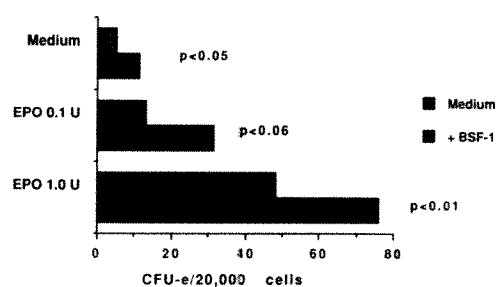


Fig 3. Role of BSF-1 in the stimulation of CFU-e. Cultures were either unstimulated or stimulated with EPO alone or in combination with BSF-1 (30 U/mL). Erythroid clusters were counted in benzidine-stained preparations after 2.5 to three days of incubation. Values represent the mean of three different experiments.

DISCUSSION

BSF-1 was originally described as a lymphokine with multiple actions on B lymphocytes.¹⁻⁷ Recently it was

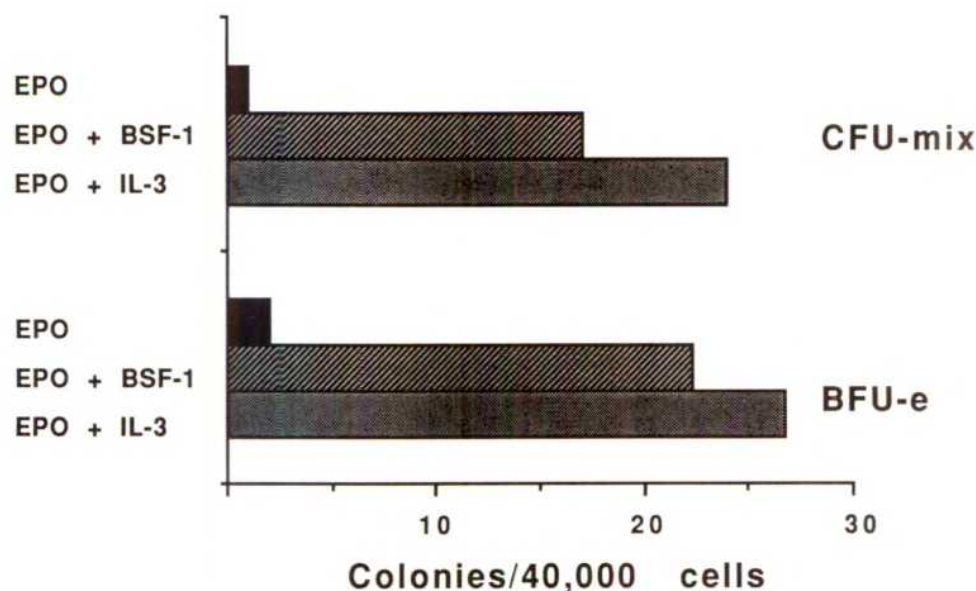


Fig 4. Role of BSF-1 in the stimulation of CFU-mix and BFU-e. Cultures were stimulated with either EPO alone or in combination with BSF-1 (30 U/mL) or IL 3 (50 U/mL). Mixed and pure erythroid colonies were counted in preparations stained with benzidine or for acetylcholinesterase after seven days of incubation. Mixed colonies contained mature erythroid cells and at least one other cell type (megakaryocytes and/or granulocytes/macrophages). Values represent the mean of four different experiments.

reported that the proliferation of T cell lines and mast cell lines is also affected by BSF-1.⁸⁻¹¹ The results presented in this study further extend the known activities of BSF-1 and provide evidence that it stimulates a broad spectrum of hematopoietic progenitor cells. Proliferative effects were detected on granulocyte-macrophage, erythroid, megakaryocyte, and multipotent progenitor cells. Such a wide range of activities was previously ascribed only to IL 3. In contrast to IL 3, however, the hematopoietic proliferative activities of BSF-1 were generally dependent on the presence of other factors.

A stimulatory effect of BSF-1 on proliferation of CFU-GM could only be detected in combination with G-CSF. Murine G-CSF at low-to-medium concentrations stimulates only a portion of the CFU-GM population to form colonies of small, mature granulocytes. G-CSF is also a potent granulocytic differentiation factor and it can initiate proliferation of multipotential and erythroid progenitor cells but is unable to sustain continued proliferation of these cells to result in colony formation.⁴⁴ The synergistic effect of BSF-1 with G-CSF could be explained by one of several mechanisms. It is possible that (1) BSF-1 enhances responsiveness of CFU-

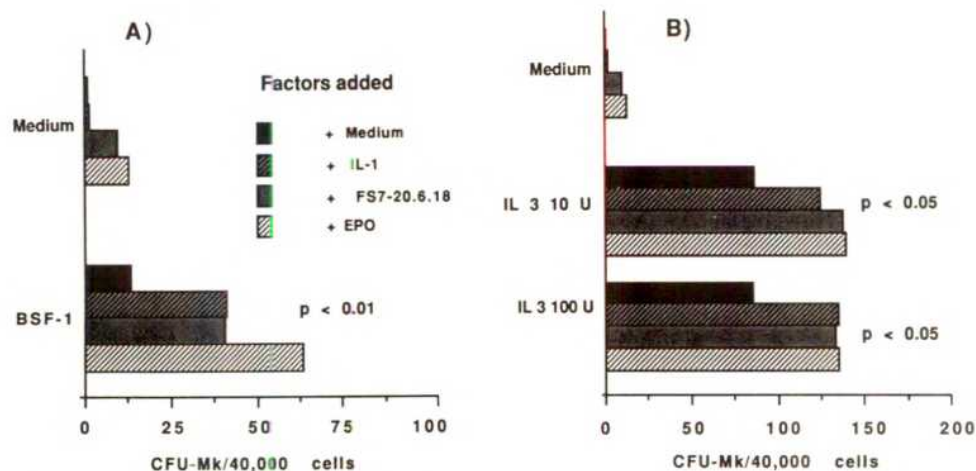


Fig 5. Role of BSF-1 in the stimulation of CFU-Mk. Cultures were either unstimulated or stimulated with BSF-1 (30 U/mL) (A) or IL 3 (10 U/mL) or IL 3 (100 U/mL) (B). To these cultures, medium alone, IL 1 (5 U/mL), FS7-20.6.18 CM 5% or EPO (2.0 U/mL) were added. Mk colonies were counted in acetylcholinesterase-stained preparations after seven days of incubation. Values represent the mean of three different experiments. The differences in colony number between cultures stimulated with BSF-1 or IL 3 alone and cultures stimulated with BSF-1 or IL 3 and one of the additional factors (IL 1, FS7-20.6.18, or EPO) were analyzed by the two-tailed Dunnett test. The *P* values for the comparison of BSF-1 alone and BSF-1 plus each of the costimulants (individually or as a group) were <0.01 . For the similar comparison for IL 3 (10 U/mL) or IL 3 (100 U/mL), the *P* values were all <0.05 .

Table 3. Lack of Synergy Between IL 3 and BSF-1 on CFU-Mk

	Number of Megakaryocytic Colonies	
	-ESF-1	+BSF-1
IL 3 None	1.5 ± 1.4*	11.6 ± 5.7
1 U	44.5 ± 17.5	51.5 ± 16.4
5 U	78.0 ± 27.8	73.6 ± 22.0
10 U	87.0 ± 22.8	78.5 ± 25.0
100 U	83.0 ± 21.0	82.5 ± 17.7

*Values represent the mean number ± SD of three different experiments.

GM to G-CSF, possibly by increasing the expression of receptors for G-CSF; (2) BSF-1 prevents terminal differentiation of progeny of CFU-GM and augments, therefore, their proliferation; or (3) G-CSF induces responsiveness of myeloid cells to BSF-1. In preliminary experiments we observed that BSF-1 can sustain the survival of CFU-GM in agar or liquid culture over several days (data not shown). Furthermore, a proliferation of macrophage colonies is also induced by BSF-1 in presence of G-CSF, a factor predominantly specific for cells of the granulocyte lineage.⁴⁴ These observations and the finding that the myeloid cell line DA-1 expresses BSF-1 receptors⁴⁵ support the possibility that BSF-1 directly stimulates CFU-GM.

Clear evidence for a direct effect of BSF-1 on progenitor cells is provided by its activity on BFU-e and CFU-mix. It is generally accepted that EPO represents the growth factor for the more mature erythroid progenitor cell, CFU-e, whereas primitive erythroid progenitor cells, BFU-e, require another activity, frequently designated as "burst-promoting activity," for proliferation. This activity has previously been shown to be provided by IL 3,¹⁵ erythroid potentiating activity (EPA),²³ and to some extent by G-CSF⁴⁴ and GM-CSF.^{17,19,20} IL 3 promotes proliferation of BFU-e and CFU-mix. Progeny of such IL 3-stimulated BFU-e and CFU-mix can then be induced by EPO to further growth and differentiation into mature erythrocytes. However, EPO does not appear to be necessary for initial proliferation of the immature progenitors.^{15,22,38} Therefore it has been suggested that EPO receptors are expressed on intermediate cells between BFU-e and CFU-e. We demonstrate here that BSF-1 and EPO together can also stimulate the growth of BFU-e to form erythroid colonies and of CFU-mix with a predominant differentiation toward erythroid and megakaryocytic cells. Since EPO by itself is believed to have no capacity to stimulate BFU-e and

CFU-mix, BSF-1, like IL 3, must be another primary stimulatory factor that prepares the progenitor cells to respond to EPO. In contrast to IL 3, however, BSF-1 alone does not appear to stimulate sustained proliferation of BFU-e and of CFU-mix, but EPO and BSF-1 are both required during the entire culture period for optimal growth of erythroid colonies. It can therefore be assumed that BSF-1 works mainly as an activation factor on BFU-e and CFU-mix and renders them sensitive to EPO, possibly by inducing the expression of functional EPO receptors.

In addition to its activity on BFU-e, BSF-1 enhances the proliferation of CFU-e in the presence of suboptimal and of optimal amounts of EPO. The enhancing effect of BSF-1 on the response of CFU-e and BFU-e to EPO resembles the activity of EPA on human and mouse erythroid progenitors. EPA has recently been purified from a HTLV-II-infected human T cell line and has been molecularly cloned.^{23,24} A comparison of the cDNA sequences of EPA²⁴ and the human analogue of BSF-1⁴⁶ demonstrates that these are distinct entities. EPA also appears to differ from BSF-1 in that its described activities are limited to erythroid lineage cells.²³

In assays for megakaryocytic progenitor cells, we found that BSF-1 exerted striking effects on colony formation. Previous studies had indicated that murine CFU-Mk are regulated in vitro by Mk-CSF and Mk potentiating activity. Mk-CSF appears to act directly on CFU-Mk cells and is now considered to be identical with IL 3²⁶; GM-CSF has also some activity on CFU-Mk.¹⁶ Mk potentiating activity (Mk-PA) is less well defined; it enhances the number and size of Mk colonies in the presence of IL 3 and acts on immature megakaryocytes rather than on CFU-Mk. Mk-PA is found in several crude supernatants from bone, bone marrow, lung and spleen cells,⁴⁷ and in supernatants of the macrophage line P388D1²⁶; recently a T cell hybridoma designated FS7-20.6.18 has been described to produce MkPA.²⁷

IL 3 by itself stimulates the production of a large number of Mk colonies. By contrast, BSF-1 alone induced only sparse small groups of megakaryocytes. Nevertheless both IL 3 and BSF-1 show synergy with the MkPA found in FS7-20.6.18 CM. Furthermore we showed that IL 1 and EPO synergize with IL 3 and thus should be regarded as having Mk-PA. Both these agents also synergize with BSF-1. This suggests that BSF-1, although stimulating by itself the appearance of only a small number of Mk colonies, shares some activity with IL 3 on CFU-Mk. The finding that BSF-1 and IL 3 do not synergize with one another also supports the concept that

Table 4. Depletion of B220⁺ Cells Does Not Inhibit Hemopoietic Progenitor Cell Proliferation Induced by BSF-1

CFU-GM			BFU-e/CFU-mix			CFU-Mk		
Growth Factor	+ B Cells	- B Cells	Growth Factor	+ B Cells	- B Cells	Growth Factor	+ B Cells	- B Cells
G-CSF 50	10/0*	21/0	EPO	0/0†	1/0	BSF-1	9	23
G-CSF 200	16/9	28/14	EPO + BSF-1	17/8	26/11	BSF-1 + IL 1	26	45
G-CSF 50 + BSF-1	29/10	42/25	EPO + IL 3	9/10	13/17	BSF-1 + T-CM‡	27	57
G-CSF 200 + BSF-1	25/22	34/66				BSF-1 + EPO	36	55
IL 3	ND/66	ND/135				IL 3	36	83
						IL 3 + T-CM	52	113

*The first value represents the number of clusters (10-40 cells); the second value represents the number of colonies (>50 cells).

†The first value represents the number of BFU-e; the second value represents the number of mixed erythroid colonies.

‡T-CM = FS7-20.6.18 CM.

they have a similar activity. Thus we would identify BSF-1 as having some Mk-CSF activity.

The MkPA of pure IL 1 and EPO have not previously been described. However, supernatants of the P388D1 cell line, a major component of which is IL 1, have been described to enhance Mk colony formation.²⁶ Our experiments using recombinant IL 1 confirm that the megakaryocyte-enhancing activity in P388D1 supernatant is most likely IL 1. Crude preparations of EPO have been used as primary stimulus of CFU-Mk⁴⁸ and as potentiating factor together with IL 3 (D. Geissler, manuscript in preparation). In another study a small level of Mk stimulatory activity was found in only one of four preparations of biochemically purified EPO.⁴⁹ We here present clear evidence that purified, recombinant EPO has an effect on megakaryocyte colony formation in the presence of IL 3 or BSF-1. Small but constant numbers of Mk colonies were also detected in cultures stimulated with EPO alone, demonstrating a direct effect of EPO at least on a subpopulation of CFU-Mk.

In conclusion, BSF-1 affects the *in vitro* proliferation of myeloid, erythroid, megakaryocyte, and multipotent progenitor cells. The nature of action on these hemopoietic cells is not yet known. It can be assumed that BSF-1 works directly on the progenitor cell population, since the depletion of adherent cells, T cells, and B cells did not result in a diminished colony proliferation. Active cell proliferation and colony formation, however, can only be observed when BSF-1 is combined with an additional factor, a property that has also been observed for BSF-1 as B cell^{2,3} and mast cell-stimulating factor^{8,9,11} and in its ability to stimulate proliferation by resting T cells.⁵⁰ The factors costimulating with BSF-1 are G-CSF for CFU-GM; EPO for CFU-e, BFU-e, and CFU-mix; and factors with MkPA for CFU-Mk proliferation. All these proteins are regarded to influence predominantly later stages of hemopoiesis, whereas certain progenitor cells (BFU-e, CFU-mix, and most CFU-Mk) do not respond to their respective factors alone. However, the

combination of these factors with BSF-1 results in a clonal proliferation and colony formation *in vitro*, suggesting a primary effect of BSF-1 on such progenitor cells. It can therefore be hypothesized that BSF-1 represents an activation factor for early hemopoietic progenitor cells and prepares the progenitor cells to respond to specific growth or differentiation factors. The effects of BSF-1 on hemato-poietic progenitor cells are reminiscent of a recently described pre-B cell stimulatory activity that has synergistic effects with IL 3, GM-CSF, and M-CSF.⁵¹ However, anti-BSF-1 antibody does not neutralize this activity, suggesting that this pre-B cell stimulating factor and BSF-1 are different entities.⁵¹

The concepts of action of BSF-1 on hematopoietic progenitor cells are clearly in keeping with the capacity of BSF-1 to act on resting B cells to enhance their subsequent responsiveness to anti-IgM antibodies^{2,3} and to LPS and with the costimulatory action of BSF-1 and phorbol esters to render T cells responsive to interleukin 2 (IL 2).⁵⁰ However, BSF-1 also possesses clear growth-promoting activities on T cells⁵⁰ and B cells.⁵² It stimulates DNA synthesis in T cell blasts and certain long-term T cell lines⁸⁻¹⁰ to enter the S phase of the cell cycle by an IL 2-independent mechanism. BSF-1 may prove to act as both a physiologic activation factor and growth factor in the regulation by T cells of hematopoietic cell growth.

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Lactoferrin Binding by Leukemia Cell Lines

By Yasuaki Yamada, Tatsuhiko Amagasaki, Donald W. Jacobsen, and Ralph Green

Monocytes and macrophages have receptors for the iron-binding protein lactoferrin. Lactoferrin acts as a potent inhibitor of granulocyte-macrophage colony stimulating factor production when it binds to these cells. Using a rosette assay and immunofluorescence, we have shown that cultured leukemia cells, including the human erythroid leukemia cell line K562, also have lactoferrin binding sites. The number of binding sites on K562 cells was estimated using soluble ^{59}Fe -lactoferrin. Inhibition studies demonstrate that lactoferrin binding sites are distinct and unrelated to receptors for transferrin or the Fc portion of IgG, which are present on K562 cells. However, electrostatic

forces may be important for lactoferrin binding, since other polycationic proteins (eg, protamine) inhibit lactoferrin binding. Prior treatment of K562 cells with trypsin nearly abolishes lactoferrin binding. However, these cells recover their ability to bind lactoferrin when trypsin is removed. Unlike transferrin receptors, the expression of lactoferrin binding sites is not regulated by cellular iron status. Cytosine arabinoside arrests the proliferation of K562 cells and simultaneously leads to a reduction in lactoferrin surface binding, suggesting that lactoferrin binding may be dependent on cell proliferation.

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LACTOFERRIN,* a cationic glycoprotein that binds two atoms of ferric iron per molecule,¹ is present in high concentration in milk (1 mg/mL),^{2,3} other body secretions,^{4,5} and in secondary granules of myeloid cells (3 $\mu\text{g}/10^6$ neutrophils),^{6,8} but is scarcely detectable in the serum (less than 1 $\mu\text{g}/\text{mL}$).⁹ It has many biochemical similarities to transferrin,¹⁰⁻¹² which is present in plasma (2 to 4 mg/mL)¹³ and other body fluids. A major biological role for transferrin as an iron transport and delivery protein has been defined. Immature erythroid cells, activated lymphocytes, and neoplastic cells, which require iron for hemoglobin synthesis or replication, take up iron efficiently through the specific receptor for transferrin.¹⁴⁻¹⁸ On the other hand, the biological significance of lactoferrin remains obscure. One of its roles is in the regulation of normal myelopoiesis. Lactoferrin binding to the surface of monocytes through a putative specific receptor results in suppression of the release of granulocyte-macrophage colony stimulating factor.¹⁹ Another suggested role is as a bacteriostatic or bactericidal agent.^{4,20} Apo-lactoferrin depresses the multiplication of iron-dependent microbial strains. Little is known about the relation between lactoferrin and neoplastic cells. By applying a rosette-

forming assay that was developed in our laboratory,²¹ we have found that neoplastic cells also express lactoferrin binding sites similar to those reported for monocytes or macrophages. We report here the nature of lactoferrin binding to cultured leukemia cells.

MATERIALS AND METHODS

Cell preparation. K562 cells, a human erythroid leukemia cell line, were used to analyze the kinetics of lactoferrin binding. Cells were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 0.25 $\mu\text{g}/\text{mL}$ fungizone (Irvine Scientific, Santa Ana, CA). MOLT-4 cells and CCRF-CEM cells (human T lymphoblastic leukemia cell lines), U937 cells (human histiocytic lymphoma cell line), HL-60 cells (human acute promyelocytic leukemia cell line), CCF-Y1 cells (a human B cell line established in our laboratory from a patient with Ph¹ negative juvenile chronic myelocytic leukemia), Raji cells (human Burkitt lymphoma cell line), and L1210 cells (mouse B lymphocytic leukemia cell line) were also used and were grown under similar conditions but with 10% fetal bovine serum medium. All experiments were conducted using cells at growth densities of 3 to 5 $\times 10^5$ cells/mL (logarithmic phase). Cells were washed once with RPMI 1640 + 0.5% bovine serum albumin (fraction V; Sigma Chemical Co, St Louis), resuspended in the same medium at a cell density of 1 $\times 10^6/\text{mL}$, and used for rosette formation.

Mononuclear cells were separated from heparinized normal blood by a density-gradient centrifugation technique using Ficoll-Hypaque (FH; Pharmacia, Piscataway, NJ). Cells were then passed through a nylon-wool column²² to enrich T lymphocytes. The resultant T lymphocytes were activated by stimulation with concanavalin A (Con A; Sigma) for 96 hours and evaluated for lactoferrin binding.

Lactoferrin and transferrin. Purified human apo-lactoferrin from colostrum (Calbiochem-Behring, La Jolla, CA) and human apo-transferrin (Sigma) were saturated with iron according to the method described by Bates and Schlabach.^{21,23} Iron saturation was confirmed spectrophotometrically by an increase in absorbance at 460 nm ($A_{460}/A_{280} = 0.045$ to 0.048 for fully saturated transferrin).²⁴ Monoclonal antibodies (MoAbs) against the human transferrin receptor (42/6 and B3/25)²⁵ were generously supplied by Dr Ian Trowbridge (Salk Institute, La Jolla, CA). OKT9 antihuman transferrin receptor antibody was purchased from Ortho Diagnostic Systems (Raritan, NJ).

Lactoferrin coating of bovine red blood cells and rosetting procedure. Lactoferrin was coated on bovine red blood cells (B-RBC) using CrCl_3 according to the method described originally for the protein A hemolytic plaque assay²⁶ and modified subsequently by

*Lactoferrin and transferrin refer to the diferric forms, unless otherwise stated.

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this laboratory for transferrin-coated B-RBC.²¹ Successful coupling of lactoferrin on B-RBC was confirmed with fluorescein isothiocyanate (FITC)-labeled antilactoferrin antibody (Cappel Worthington Biochemicals, Malvern, PA). Apo-lactoferrin, transferrin, or albumin-coated B-RBC were prepared similarly, using the same concentrations of protein. Cultured neoplastic cells or T lymphocytes (1×10^6 /mL in 0.25 mL) were mixed with 0.1 mL of lactoferrin, transferrin, or albumin-coated B-RBC (0.5% packed cell volume) in U-bottom 12 $\times$ 75-mm tubes (Falcon #2054, Oxnard, CA). The mixtures were centrifuged at $30 \times g$ for three minutes and then incubated at 37°C for 45 minutes (except for the time-course study). The pellets were carefully resuspended, and rosette formation was estimated visually using a hemocytometer. More than 200 cells were counted, and those cells binding more than three B-RBC on their surface were considered positive for rosette formation. All experiments were performed in triplicate.

Immunofluorescence assay. Lactoferrin binding was also evaluated by an immunofluorescence assay using soluble lactoferrin. After washing three times with PBS, 1×10^6 pelleted cells were incubated with 100 μ L of lactoferrin solution (50 μ g/mL in PBS) at 4°C for 60 minutes. The treated cells were washed three times with PBS, and then incubated with 50 μ L of a 1/20 dilution of FITC-conjugated rabbit antihuman lactoferrin antibody (F(ab')₂ fraction, Cappel Labs). After washing three times with PBS, the labeled cells were examined using a fluorescence-activated cell sorter.

⁵⁹Fe-lactoferrin binding assay. Apo-lactoferrin was saturated with ⁵⁹Fe (⁵⁹FeCl₃, New England Nuclear, Boston) by the method used for preparing nonradiolabeled diferric-lactoferrin.^{21,23} K562 cells (1×10^6) suspended in Hank's balanced salt solution (HBSS) were incubated at 4°C for 60 minutes with various concentrations of ⁵⁹Fe-lactoferrin. After incubation cells were washed three times with HBSS, and radioactivity was measured in a gamma spectrometer.

Benzidine staining. K562 cells were cultured in the presence of 3.6×10^{-7} mol/L cytosine arabinoside (Sigma) to induce erythroid differentiation²⁷ for assessment of differentiation-associated change in lactoferrin binding ability. Hemoglobin synthesis in these cells was estimated by benzidine staining.²⁷ The proportion of cells that synthesized hemoglobin (stained blue) was assessed by light microscopy.

Other chemicals. Deferoxamine mesylate (Desferal, CIBA Pharmaceutical Co, Summit, NJ) or ferrie ammonium citrate (Sigma) were dissolved in distilled water and added to culture media at final concentrations of 1×10^{-5} mol/L and 10 μ g/mL respectively to determine the effect of iron content of the culture solution on the expression of transferrin receptor and lactoferrin binding sites. Cells treated with 0.25% trypsin solution (Irvine Scientific) for one minute at 37°C, cells treated with 2 mg/mL of deoxyribonuclease I or ribonuclease A (DNase or RNase, Sigma) for one hour at room temperature, and cells treated with 0.1 U/mL of neuraminidase (Calbiochem-Boehringer) for 20 minutes at 37°C were used to assess the susceptibility of lactoferrin binding sites to digestion by these enzymes. Human IgG (Sigma) was incubated for 30 minutes at 60°C to prepare heat-aggregated IgG for use in experiments to study inhibition of rosette formation. Human milk lysozyme and salmon protamine (Sigma) were also used to study the specificity of lactoferrin binding.

RESULTS

Time-course study of rosette formation. Mixtures of K562 cells and lactoferrin-, apo-lactoferrin- or albumin-coated B-RBC were incubated for up to 90 minutes at 37°C. Rosette formation with lactoferrin-coated B-RBC increased rapidly and reached a plateau after 45 minutes incubation (Fig 1). Rosette formation was lower with apo-lactoferrin-

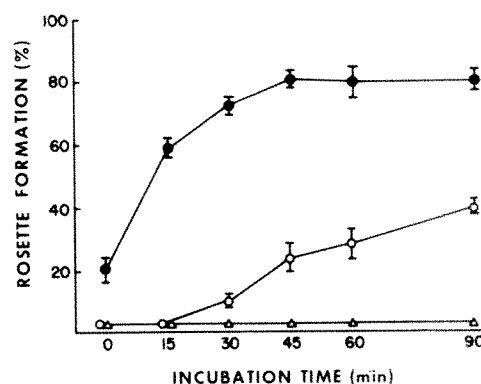


Fig 1. Time course of rosette formation. K562 cells were incubated with lactoferrin- (●—●), apo-lactoferrin (○—○), or albumin- (△—△) coated B-RBC. Each point is the mean of three assays; vertical bars indicate standard deviations.

coated B-RBC. Control albumin-coated B-RBCs did not form rosettes throughout the incubation time.

Determination of lactoferrin binding sites by Scatchard analysis. ⁵⁹Fe-radiolabeled lactoferrin showed saturable binding to K562 cells (Fig 2). A Scatchard plot analysis of these data showed maximum binding of 4.9×10^7 molecules per cell; the dissociation constant was 7.4×10^{-6} mol/L. A competition assay using a tenfold excess of unlabeled lactoferrin was also attempted but was unsuccessful due to the technical problem caused by cell aggregation at lactoferrin concentrations greater than 2.5 mg/mL.

Specificity of rosette-forming assay for detection of lactoferrin binding sites. To test the specificity of the rosette-forming assay, K562 cells were preincubated with either soluble lactoferrin, transferrin, or MoAbs against transferrin receptor for 30 minutes at room temperature before addition of lactoferrin-coated B-RBC. Rosette formation was inhibited by prior treatment with soluble lactoferrin in a dose-dependent manner (Fig 3). Soluble apo-lactoferrin also inhibited rosette formation almost to the same extent as did soluble lactoferrin (Table 1). However, neither transferrin nor three types of MoAbs against transferrin receptor inhibited rosette formation with lactoferrin-coated B-RBC (Table 1); all inhibited rosette formation with transferrin-coated B-RBC at the same concentrations.²¹ Heat-aggregated IgG at a concentration of 64 μ g/mL, which is sufficient to fully saturate all Fc receptors on K562 cells,²⁸ did not inhibit rosette formation (Table 1). Since lactoferrin is a cationic protein (pI = 8.7),²⁹ nonspecific electrostatic binding to the cell surface membrane was considered. To investigate this possibility, we used two kinds of cationic proteins in competition studies: human milk lysozyme and salmon protamine. Lysozyme caused no inhibition, but protamine substantially inhibited rosette formation. Furthermore, neuraminidase, an enzyme that cleaves membrane surface sialic acid resulting in decreased cell surface negative charge, also partially inhibited rosette formation.

Trypsin sensitivity of lactoferrin binding sites. K562 cells were examined for rosette formation with lactoferrin-coated B-RBC before and after digestion with trypsin. In three experiments, rosette formation decreased markedly

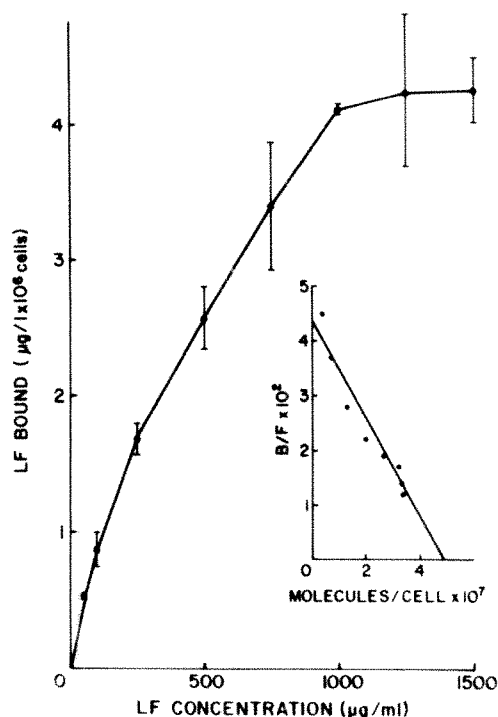


Fig 2. Dose-response binding of ⁵⁹Fe-radiolabeled lactoferrin to K562 cells. The mean of three experiments is shown. A Scatchard plot analysis (inset), indicates that 4.9×10^7 molecules are bound to each K562 cell at saturation. The dissociation constant (K_d) derived from the concentration of free ligand at half saturation of the binding sites is 7.4×10^{-6} mol/L.

following trypsin digestion (from $82.9\% \pm 0.6\%$ to $11.8\% \pm 3.9\%$) but recovered almost to pretreatment levels following culture of the digested cells for five hours at 37°C in culture medium without trypsin ($74.6\% \pm 2.0\%$). Trypsin sensitivity of lactoferrin binding by MOLT-4 cells was also shown using the immunofluorescence assay (Fig 4). Binding

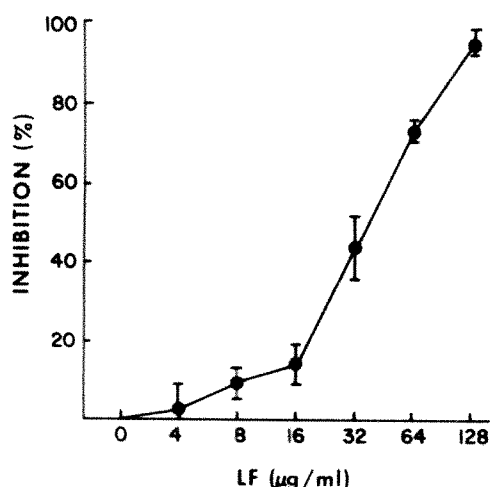


Fig 3. Inhibition of rosette formation by soluble lactoferrin. K562 cells were pretreated with soluble lactoferrin before rosette formation. Each point is the mean of three assays; vertical bars indicate standard deviations.

Table 1. Specificity of Rosette-Forming Assay

Treatment of K562 Cells*	Inhibition of Rosette Formation (%) Mean $\pm$ SD (n = 3)
Lactoferrin (64 $\mu\text{g}/\text{mL}$)	74.0 ± 1.8
Apo-lactoferrin (64 $\mu\text{g}/\text{mL}$)	77.9 ± 3.6
Transferrin (64 $\mu\text{g}/\text{mL}$)	-1.9 ± 6.8
Apo-transferrin (64 $\mu\text{g}/\text{mL}$)	-3.1 ± 9.0
MoAbs against transferrin receptor	
42/6 (1 $\mu\text{g}/\text{mL}$)	9.1 ± 3.9
B3/25 (1 $\mu\text{g}/\text{mL}$)	3.4 ± 5.9
OKT9 (1 $\mu\text{g}/\text{mL}$)	5.4 ± 8.7
Heat-aggregated human IgG (64 $\mu\text{g}/\text{mL}$)	-1.5 ± 3.3
Cationic proteins	
Human milk lysozyme (64 $\mu\text{g}/\text{mL}$)	-1.9 ± 1.0
Salmon protamine (64 $\mu\text{g}/\text{mL}$)	44.6 ± 8.9
Neuraminidase (0.1 U/mL)	31.5 ± 4.0

*K562 cells were pretreated with these agents before rosette formation for 30 minutes at room temperature except for neuraminidase treatment, which was performed at 37°C .

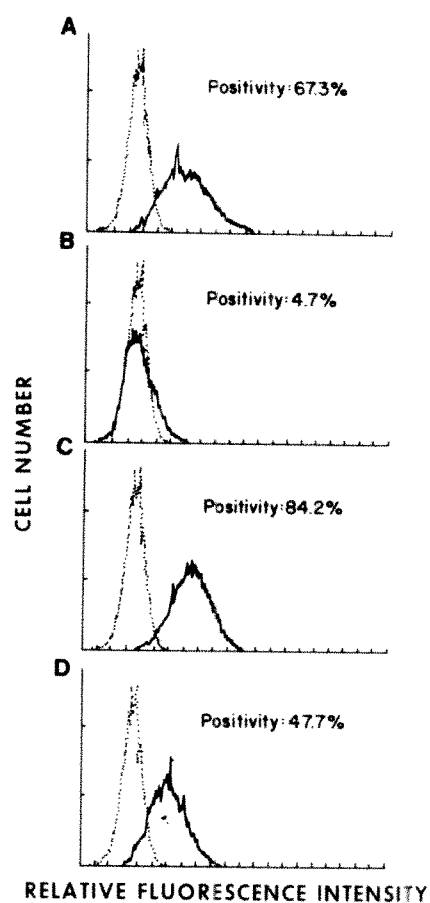


Fig 4. Trypsin sensitivity of lactoferrin binding sites. Labeled MOLT-4 cells (—) were processed on a fluorescence-activated cell sorter and compared with control cells (---) treated with FITC-conjugated antibody only. Nontreated cells (A), trypsin-digested cells (B), DNase-treated cells (C), and RNase-treated cells (D) were analyzed for lactoferrin binding.

was almost completely abrogated by trypsin digestion (Fig 4B). DNase did not remove but rather enhanced lactoferrin binding (Fig 4C). Interestingly, RNase caused slight inhibition of lactoferrin binding (Fig 4D).

Influence of iron availability on the expression of lactoferrin binding. K562 cells were cultured up to 96 hours in the presence of the iron chelator deferoxamine (1×10^{-5} mol/L) or 10 $\mu\text{g/mL}$ ferric ammonium citrate. At various times lactoferrin and transferrin binding by these cells was assessed with the rosette-forming assay. Proliferation status of K562 cells was not affected by either agent at these concentrations, and the doubling times of these cells were similar to untreated control cells. Rosette formation with transferrin-coated B-RBC increased two to three times following deferoxamine treatment but decreased to less than one-half following addition of ferric ammonium citrate (Fig 5A). However, rosette formation with lactoferrin-coated B-RBC was not influenced by these agents (Fig 5B).

Influence of cell proliferation status on expression of lactoferrin binding. K562 cells were cultured with cytosine arabinoside (C-ara) and examined for rosette formation with lactoferrin-coated B-RBC at 24-hour intervals. The culture solution was replaced by fresh media containing C-ara every other day. Cell proliferation ceased after 24 hours in C-ara medium, but the viability remained greater than 90%. The proportion of benzidine-positive cells increased daily and reached 77.7% at 144 hours, indicating maturation to hemoglobin-producing cells. There was a corresponding inverse decline in rosette formation rate (Fig 6). This decrease in lactoferrin binding could be curtailed by removing C-ara from the culture media.

Lactoferrin binding sites on various cells. Expression of lactoferrin binding sites was examined on other neoplastic cell lines as well as on normal blood lymphocytes before and after activation with Con A (Table 2). The immunofluorescence assay was always more sensitive than the rosette-forming assay. Four (K562, MOLT-4, L1210, and CCF-Y1) of eight cell lines examined showed abundant lactoferrin binding sites. The rosette-forming assay failed to detect lactoferrin binding sites on activated T lymphocytes, but the immunofluorescence assay showed that approximately one half of activated T lymphocytes display lactoferrin binding.

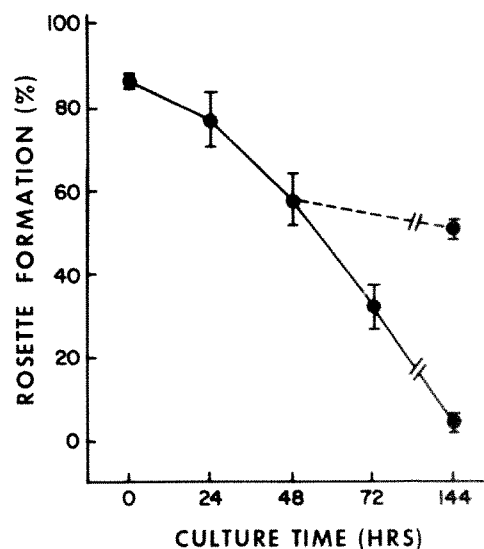


Fig 6. Effects of C-ara on the expression of lactoferrin binding sites. Rosette formation was performed on K562 cells cultured in the presence of C-ara (●—●). For some cells C-ara was removed at 48 hours, and culture was continued without C-ara (○—○). Each point is the mean of three assays; vertical bars indicate standard deviations.

DISCUSSION

Although more than 25 years have passed since the iron-binding protein lactoferrin was found in milk,³ its biological functions are still poorly understood. Receptor-like binding of lactoferrin to human peripheral monocytes has been described.^{30,31} Furthermore, it has been reported that lactoferrin has an inhibitory effect on the release of granulocyte-macrophage colony stimulating factor (GM-CSF) from monocytes and macrophages.¹⁹ Few studies have been done on the relation between lactoferrin and neoplastic cells. Two recent articles have shown that lactoferrin is an essential nutrient for the growth of neoplastic cell lines in serum-free medium. Growth stimulation of Bri 7 cells, a human B lymphocytic leukemia cell line, was greater with lactoferrin than with transferrin.³² Similarly, growth of HT29, a human colon adenocarcinoma cell line, was also stimulated by

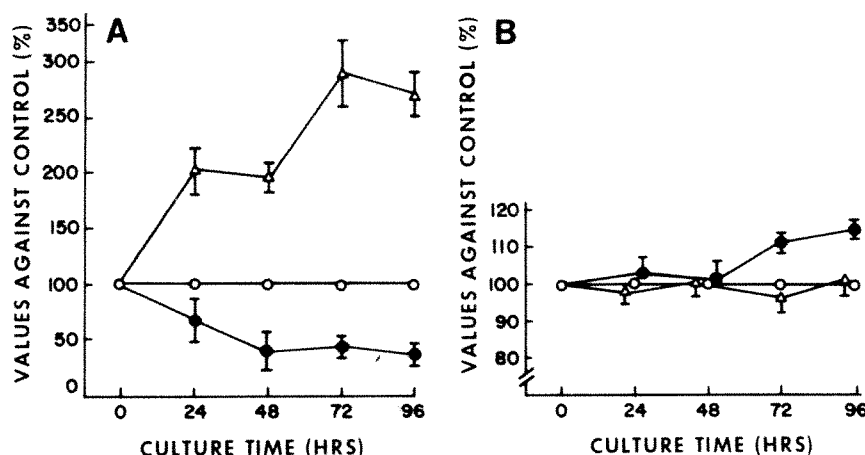


Fig 5. Influence of iron content of culture media on the expression of transferrin receptor (A) and lactoferrin binding sites (B). K562 cells were cultured with deferoxamine (△—△) or ferric ammonium citrate (●—●) for varying times up to 96 hours and compared with control cells (○—○) for their transferrin and lactoferrin binding ability. Each point is the mean of three assays; vertical bars indicate standard deviations.

Table 2. Lactoferrin Binding by Neoplastic Cell Lines and Normal T Lymphocytes

Cells	Rosette Formation % Mean $\pm$ SD (n = 3)	Immunofluorescence Assay (%)
K562	90.0 $\pm$ 3.8	97.7
MOLT-4	21.3 $\pm$ 5.2	67.3
L1210	28.9 $\pm$ 3.3	ND
CCF-Y1	13.0 $\pm$ 1.4	91.8
CCRF-CEM	2.9 $\pm$ 0.5	ND
HL-60	0.7 $\pm$ 0.7	9.7
U937	0	14.0
Raji	0	4.9
Resting T lymphocytes	0	2.8
Mitogen-stimulated T lymphocytes	0	55.4

lactoferrin.³³ The effect was more pronounced in the presence of iron. These reports led us to study lactoferrin binding sites on neoplastic cells.

In the present studies we used a rosette-forming assay to detect lactoferrin binding. Rosette formation rate was greater with lactoferrin-coated than with apo-lactoferrin-coated B-RBC, suggesting a higher binding affinity of iron-saturated lactoferrin. Although differences in the quantity of protein coating cannot completely be excluded, it is unlikely that this is the cause of the discrepancy, since the same concentration of lactoferrin or apo-lactoferrin was used for coating. By analogy, iron-saturated transferrin has higher binding affinity to its receptor than apo-transferrin.³⁴⁻³⁶ The transferrin molecule becomes more compact and spherical after it has bound iron,^{34,37,38} and this may be advantageous for receptor binding. On the other hand, rosette formation with lactoferrin-coated B-RBC was inhibited by soluble lactoferrin irrespective of its iron content, suggesting that low affinity binding is sufficient to suppress rosette formation. However, lactoferrin-coated B-RBC rosette formation was not inhibited by transferrin, MoAbs against the transferrin receptor, or heat-aggregated human IgG. This indicates that lactoferrin occupies binding sites different from those for either the transferrin receptor or the Fc receptor for IgG, which are both present on K562 cells.²⁸

The number of lactoferrin binding sites on K562 cells was estimated by Scatchard analysis using ⁵⁹Fe-lactoferrin. Binding of ⁵⁹Fe-lactoferrin was saturable, and the number of binding sites per cell (4.9×10^7), though high, agrees with figures reported for adherent mononuclear cells (3.3×10^7 per cell) and nonrosetting lymphocytes (2.5×10^7 per cell) prepared from normal human blood.³⁰ Because of problems with cell aggregation that we encountered at high lactoferrin concentrations, it was not possible to eliminate nonspecific binding, so that our figure is likely an overestimate of the number of binding sites.

Since lactoferrin is a cationic protein (pI = 8.7),²⁹ we considered that binding to the negatively charged cell surface might be of a nonspecific electrostatic nature. We therefore pretreated K562 cells with two other cationic proteins to block surface-negative charges. The more cationic lysozyme (pI = 10.5 to 11.0)³⁹ did not inhibit lactoferrin-B-RBC rosette formation. However, protamine caused considerable

inhibition, although the inhibitory activity was less than that of soluble lactoferrin. Sialic acid, one of the major sources of surface negative charge, was removed by neuraminidase treatment⁴⁰ of K562 cells and resulted in a decrease of rosette formation. Taken together these findings suggest that electrostatic factors are intimately involved in lactoferrin binding to cells. Similar electrostatic factors have been implicated in the binding of lactoferrin to macrophages and liver reticuloendothelial cells. Lactoferrin binds to alveolar macrophages in competition with the other cationic neutrophil granule glycoproteins, elastase and cathepsin G.⁴¹ Removal of sialic acid from lactoferrin increases its binding affinity to mouse peritoneal macrophages.⁴² Moreover, based on a study of lactoferrin uptake by the liver, the possible existence of common binding sites for certain cationic proteins on liver reticuloendothelial cells has been postulated.⁴³ The lactoferrin binding sites we have demonstrated on neoplastic cell lines are similar in some respects to the lactoferrin binding sites described on macrophages and liver reticuloendothelial cells. However, there are also differences, such as the sensitivity to trypsin digestion. We found marked sensitivity to trypsin digestion of lactoferrin binding by neoplastic cells. In contrast, lactoferrin binding by human peripheral blood monocytes is somewhat resistant to trypsin digestion.⁴⁴ A further difference is the sensitivity to DNase and RNase. Lactoferrin binding sites on neoplastic cells were resistant to DNase but were partially sensitive to RNase, suggesting that lactoferrin binding sites may be RNase-susceptible acidic groups present on the cell surface.⁴⁰ In contrast to neoplastic cells, lactoferrin binding sites on normal human monocytes are sensitive to DNase treatment.⁴⁴

Transferrin receptor expression of cultured cells is regulated by the iron concentration of the culture medium. An iron chelator, deferoxamine, increases the number of transferrin receptors, whereas excess iron decreases transferrin receptor expression.^{24,45,46,47,48} We confirmed this observation by finding a good inverse correlation between iron supply and transferrin-receptor expression using our rosette-forming assay. Lactoferrin shows close structural homology to transferrin at the iron binding site, but unlike transferrin, lactoferrin binding to cells was not influenced by cellular iron status. This finding supports the view that K562 cells cannot take up iron from lactoferrin even though cells can bind and internalize lactoferrin (unpublished observation).

Four of eight leukemic cell lines as well as activated T lymphocytes showed lactoferrin binding, but resting T lymphocytes did not. Similarly, receptor-like binding of lactoferrin to fresh leukemia cells, but not to normal lymphocytes or platelets has been reported.⁴⁹ Furthermore, we were able to demonstrate diminished expression of lactoferrin binding by C-ara treatment of K562 cells. These results seem to indicate that there is a close relationship between lactoferrin binding and cell proliferative status. Although monocytes and activated lymphocytes also have lactoferrin binding sites, analysis in conjunction with other cell markers may prove useful for the identification of leukemic cells in blood or neoplastic cells in body fluids suspected to contain metastatic tumor.

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CFU-GM Content of Bone Marrow Graft Correlates With Time to Hematologic Reconstitution Following Autologous Bone Marrow Transplantation With 4-Hydroperoxycyclophosphamide-Purged Bone Marrow

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Autologous bone marrow transplants (BMTs) can repopulate the hematologic system of patients treated with marrow-ablative chemotherapy and/or radiotherapy. However, treatment of the bone marrow graft to eliminate residual tumor cells prior to reinfusion can delay the return of peripheral blood elements, presumably from damage to or loss of hematopoietic stem cells responsible for hematologic recovery. To develop a model predictive of hematologic recovery, we studied the progenitor cell contents of 4-hydroperoxycyclophosphamide (100 $\mu\text{g}/\text{mL}$)-purged bone marrow grafts of 40 consecutive patients undergoing autologous BMT at this center. Granulocyte-macrophage colonies (CFU-GM) were grown from all grafts after treat-

ment with this chemotherapeutic agent, but erythroid (BFU-E) and mixed (CFU-GEMM) colonies were grown from only 44% and 33% of the grafts respectively. The recovery of CFU-GM after purging ranged from 0.07% to 23%. The logarithm of CFU-GM content of the treated grafts was linearly correlated with the time to recovery of peripheral blood leukocytes ($r = -0.80$), neutrophils ($r = -0.79$), reticulocytes ($r = -0.60$), and platelets ($r = -0.66$). The CFU-GM content of purged autologous bone marrow grafts may reflect the hematopoietic stem cell content of the grafts and thus predict the rate of hematologic recovery in patients undergoing autologous BMT.

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AUTOLOGOUS bone marrow transplantation (BMT) permits the administration of intensive cytotoxic regimens to patients with a variety of malignancies who do not have human leukocyte antigen (HLA)-matched bone marrow donors.¹⁻⁵ For tumors that originate in or frequently metastasize to the bone marrow, techniques of purging residual malignant cells from the harvested bone marrow graft prior to reinfusion have been developed and used in clinical trials.⁶ This center has studied autologous BMT for the treatment of acute leukemia using bone marrow grafts incubated ex vivo with 4-hydroperoxycyclophosphamide (4-HC) before cryopreservation and subsequent reinfusion.^{7,8} In a phase I trial,⁷ a concentration of 100 $\mu\text{g}/\text{mL}$ 4-HC was determined to be the maximum that would allow consistent hematologic reconstitution. Many patients regained hematopoietic function despite the absence of detectable CFU-C in the treated marrow grafts, and CFU-C determinations did not appear to be predictive of hematologic reconstitution in these patients. However, the time to reconstitution appeared to increase with increasing 4-HC concentrations, indicating that treatment of the grafts with this drug may have adversely affected cells responsible for hematologic recovery.

Ex vivo purging allows the use of agents that are too toxic to nonhematopoietic tissue or are otherwise unsuitable for in vivo administration. Since toxicity to normal human hematopoietic stem cells can be expected with the various manipulations required for tumor cell purging and graft preservation, an indicator of the bone marrow repopulating potential of such treated cells would greatly enhance our ability to evaluate clinical autologous BMT regimens.

In this report we describe the in vitro assay of CFU-GM-derived colonies from 4-HC-treated bone marrow grafts in a series of patients undergoing autologous BMT at this center. The time to hematologic reconstitution (determined by the return of peripheral blood granulocytes, reticulocytes, and platelets) showed a linear correlation to the logarithm of CFU-GM content of the treated bone marrow grafts.

METHODS

Patient selection and transplant procedures. Hematopoietic progenitor cell assays were performed on the bone marrow grafts of 40 consecutive patients undergoing autologous BMT with 4-HC-purged bone marrows at this center. Eighteen of the patients were transplanted for the diagnosis of acute nonlymphoblastic leukemia in second remission and 16 for the diagnosis of refractory Hodgkin's disease (9) or non-Hodgkin's lymphoma (7). Five patients with pediatric solid tumors and one patient with acute lymphoblastic leukemia in first remission were also harvested. The age range for these patients was 2 to 53 years with a median of 29 years. Twenty-eight of the patients were male. The details of the induction regimens used have been previously reported.^{9,10} Patients with acute nonlymphocytic leukemia, Hodgkin's or non-Hodgkin's lymphoma with bulky disease, and pediatric solid tumors (neuroblastoma, melanoblastoma, and rhabdomyosarcoma) received busulfan (4 mg/kg daily $\times$ 4) and cyclophosphamide (50 mg/kg daily $\times$ 4) for induction. The remaining patients received an induction regimen consisting of cyclophosphamide (as noted above) and total body irradiation (TBI; to 1200 rad). All patients received appropriate supportive care, including prophylactic platelet transfusions for platelet counts less than 20,000/ μL . Approval for the therapeutic

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application of autologous BMT at this center was obtained from the Joint Committee on Clinical Investigation of the Johns Hopkins Hospital and the Johns Hopkins University, and informed consent was obtained for all patients prior to bone marrow harvesting.

Marrow processing. The bone marrow grafts were collected, treated with 4-HC, cryopreserved, and thawed as previously described.^{7,8} Briefly, all bone marrows were incubated with 100 $\mu\text{g}/\text{mL}$ of 4-HC at a nucleated cell concentration of 2×10^7 cells/mL for 30 minutes at 37°C. The red cell contents of the incubation mixtures were not adjusted (the range of packed red cell volumes was 5% to 23% for this series). The cells were then resuspended for cryopreservation with 10% dimethyl sulfoxide (DMSO) in the liquid phase of nitrogen. Subsequently the cells were rapidly thawed in a 37°C water bath and immediately infused without further manipulation.

Cell culture. Aliquots of bone marrow cells were obtained from the bone marrow graft buffy coats before and after the 4-HC treatment (prior to the addition of cryoprotectants). Light-density mononuclear cells (sp gr ≤ 1.077 g/mL) from both the treated and untreated specimens were obtained by Ficoll-Hypaque (FH) separation. The cells were cultured in 1.32% methylcellulose containing alpha-medium (Gibco, Grand Island, NY), 5% phytohemagglutinin (PHA)-stimulated leukocyte-conditioned medium,¹¹ 1% bovine serum albumin (Sigma Chemical Co, St Louis), 30% fetal bovine serum (FBS, Hyclone, Logan, UT), 10^{-6} mol/L methylprednisolone, 10^{-4} mol/L 2-mercaptoethanol, and 1 U/mL erythropoietin (Amgen, Thousand Oaks, CA). Untreated cells were plated at a seeding density of 5×10^4 cells per 1-mL plate (Lux; Miles Laboratories, Naperville, IL). Treated cells were plated at multiple seeding densities from 5×10^4 to 1×10^6 cells/mL. All samples were cultured in quadruplicate and maintained at 37°C in a humidified 5% CO_2 in air atmosphere. CFU-GM-, BFU-E-, and CFU-GEMM-derived colonies were scored on an inverted microscope after 14 days of culture. The same bone marrow samples were also cultured for CFU-C as previously described.⁷ Briefly, 1×10^5 untreated, and 5×10^5 and 1×10^6 treated light-density cells were cultured in 1 mL of McCoy's medium (Gibco) containing 0.3% agar (Difco Laboratories, Detroit), 15% fetal bovine serum (FBS), and 10% human placenta-conditioned medium. Quadruplicate cultures were maintained at 37°C in a humidified 7.5% CO_2 in air atmosphere, and colonies containing >50 cells were enumerated on an inverted microscope after ten days.

Determination of bone marrow graft-progenitor cell content. The hematopoietic progenitor contents of the marrow grafts before and after incubation with 4-HC were determined by multiplying the number of colonies scored per number of cells seeded into culture by the number of nucleated cells (per kg recipient weight) within the grafts. Because the cloning efficiency of the treated cells fell to 80% of predicted at a seeding density of 10^6 cells/mL, all calculations of graft progenitor cell content were based, when possible, on the lowest seeding density giving rise to at least five colonies per plate (usually 5×10^5 cells/mL seeding density). We assumed that all progenitor cells were contained within the light-density fraction after density-gradient separation and multiplied the above product by the proportion of cells recovered within this fraction. $\text{CFU}/\text{kg} = \text{CFU}/\text{plate} \div \text{cells}/\text{plate} \times \text{cells}/\text{kg} \times \text{gradient recovery}$.

Analysis of hematologic recovery. Daily peripheral blood leukocyte counts were obtained following the reinfusion of the thawed bone marrows (day 0). When the WBC was less than $1,000/\mu\text{L}$, the WBC and granulocyte count were determined with flow cytometry (Spectrum III, Ortho Diagnostic Systems Inc, Westwood, MA). Above a WBC of $1,000/\mu\text{L}$, the WBC was determined on an automated cell counter (ELT 800, Ortho Diagnostic Systems Inc), and the granulocyte fraction was determined visually with Wright-stained peripheral blood smears. Platelet counts were determined

daily using the automated cell counter. Reticulocyte counts were measured twice weekly with the flow cytometer using acridine orange-stained specimens. The day of recovery is the first day after the reinfusion of the marrow on which the particular peripheral blood count (sustained leukocytes $\geq 1,000/\mu\text{L}$, granulocytes $\geq 500/\mu\text{L}$, reticulocytes $\geq 2\%$) is achieved. For analysis of platelet recovery we chose the time to the last platelet transfusion, which corresponded to a self-sustained peripheral blood platelet count of greater than $20,000/\mu\text{L}$ for most patients.

Statistical evaluation. The relation of the logarithm of the progenitor cell content of the bone marrow graft to the time of hematologic recovery of the recipients was evaluated by linear regression analysis and calculation of Pearson's correlation coefficient. The significance of the correlation parameters was tested by Student's *t* test.

RESULTS

The hematopoietic culture data for the various progenitor cells before and after incubation with 4-HC are shown in Table 1. The mean recovery of each progenitor cell type was similar (approximately 5%; 2% for CFU-C). However, CFU-GM-derived colonies were obtained from all bone marrows following the ex vivo incubation. BFU-E- and CFU-GEMM-derived colonies were grown from all specimens before treatment but from only 44% and 33% respectively of the specimens after treatment. CFU-C-derived colonies were cultured from only 17 (42%) of the treated bone marrow grafts.

The clinical protocols prescribed a minimum number of nucleated cells to be harvested and cryopreserved for the patients undergoing autologous BMT; therefore most patients had similar numbers of nucleated cells and CFU-GM within the bone marrow grafts prior to the purging steps. The mean CFU-GM content of the bone marrow grafts before the 4-HC incubation was $1.4 \pm 0.7 \times 10^5$ ($\pm\text{SD}$) per kg body weight (range, 0.5×10^5 to 4.0×10^5). The mean CFU-GM content after the 4-HC incubation was $7.6 \pm 13.3 \times 10^3$ ($\pm\text{SD}$) per kg with a range from 5.0×10^1 to 5.7×10^4 per kg. The recovery of CFU-GM within the graft after incubation with 4-HC ranged from 0.07% to 23%.

No relation was evident between the nucleated cell, CFU-C, BFU-E, or CFU-GEMM content of the treated bone marrow grafts and time to recovery of peripheral blood cells. The logarithm of the CFU-GM content showed a linear correlation with time to leukocytes $\geq 1,000/\mu\text{L}$, granulocytes $\geq 500/\mu\text{L}$, reticulocytes $\geq 2\%$, and time to last platelet transfusion (Figs 1 A to D). The logarithm of CFU-GM content also predicted the day of recovery when analyzed for subgroups of patients. This included the time to a WBC $\geq 1,000/\mu\text{L}$ for both the acute nonlymphocytic leukemia (ANLL) ($r = -0.79$, $n = 15$) and all lymphoma patients (Hodgkin's and non-Hodgkin's lymphomas, $r = -0.74$, $n = 11$), and time to 500 granulocytes/ μL ($r = -0.78$ and $r = -0.63$ respectively). Sufficient evaluable patient courses are not available for similar comparative analysis of time to recovery of platelets and reticulocytes. However, these data suggest that the relationship of CFU-GM to pluripotent stem cell contents of the bone marrow grafts after purging is similar for these patient groups and that the patient diagnoses do not affect the subsequent transplant course when the grafts are purged ex vivo with 4-HC. Only four patients with lym-

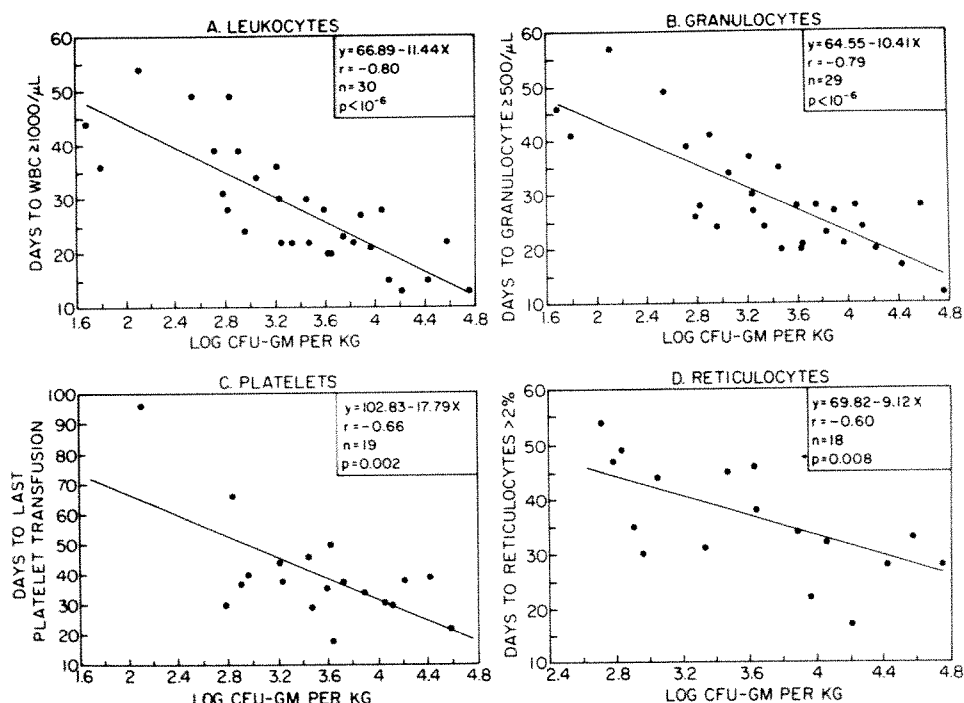


Fig 1. Number of days following bone marrow reinfusion to achieve: (A) peripheral blood total leukocyte count greater than 1,000/ μL , (B) peripheral blood granulocyte count greater than 500/ μL , (C) last platelet transfusion, and (D) peripheral blood reticulocyte concentration of greater than 2%. Correlation coefficients and the linear regression equation are shown for each curve. All slopes are significantly different from 0, as shown by the *P* values for each equation.

phoma for whom reconstitution data are available received induction with cyclophosphamide and total body irradiation (TBI), so a comparison within this particular diagnostic category for the two preparative regimens used cannot be made at this time.

Patients who could not be evaluated for reconstitution were lost to follow-up for a variety of reasons. Seven died before reaching 1,000 WBC/ μL , one patient relapsed, and one each were either not transplanted or received a "reserve" marrow (prior to time of predicted recovery) treated at a lower concentration of 4-HC. Similarly, 14 patients died while still receiving platelet transfusions, three patients relapsed, and two developed autoimmune thrombocytopenia.¹² Two of the patients who died while still requiring platelet support developed veno-occlusive disease of the liver,¹³ and one patient developed cytomegalovirus (CMV)-associated bone marrow failure¹⁴ prior to the predicted day of platelet recovery determined from the regression equation (Fig 1C). The remainder of the patients who died before white cell or platelet recovery expired prior to the day of predicted recovery. Two patients relapsed with leukemia while still receiving platelet support 83 and 116 days after the predicted day of recovery. No other factor for this delay in platelet recovery was obvious, and none of the lymphoma patients who relapsed had delayed platelet or white cell recovery.

DISCUSSION

Ex vivo manipulation of bone marrow grafts can affect the ability of these grafts to reconstitute the hematopoietic system of the recipients. In the phase I trial of purging

autologous bone marrows with 4-HC reported from this center,⁷ the time to hematopoietic recovery was related to the concentration of 4-HC to which the bone marrow grafts were exposed. Therefore an assay that demonstrates a strong correlation with the ability of the bone marrow graft to repopulate the hematopoietic system of the recipient is valuable both in terms of patient management as well as in the development or modification of purging techniques.

Other investigators have reported that the granulocyte-macrophage progenitor cell (CFU-GM) content of the autologous bone marrow graft relates either in a logarithmic fashion¹⁵ or in a step fashion² to the time to return of peripheral blood leukocytes but not platelets or reticulocytes after autologous BMT. These studies involved bone marrow grafts that were not exposed ex vivo to purging agents that could be toxic to normal committed and pluripotent progenitor cells or hematopoietic regulatory cells. In allogeneic transplantation a correlation between the CFU-GM content of the graft and hematologic reconstitution has^{16,17} and has not^{18,19} been reported. Common features of those studies that do not find a correlation are both a limited range in the number of progenitor cells infused and a usual dose of CFU-GM greater than $10^5/\text{kg}$ of recipient weight. Because our data support a model of exponential growth²⁰ of the infused bone marrow, the effects of minor (even twofold) variations in the number of CFU-GM infused may not be discernible in clinical studies. Thus patient-to-patient differences in cell loss during freezing or in the assumed FH recovery²¹ upon which our calculations are based may have little effect upon the overall correlation of progenitor cell content with hematologic reconstitution. The discrepancy

Table 1. Progenitor Cell Growth From Patient Bone Marrow

	Progenitor Cell			
	BFU-E	CFU-GM	CFU-GEMM	CFU-C
Untreated	46.7 ± 4.5*	63.0 ± 5.2	2.3 ± 0.3	15.9 ± 2.2
4-HC-treated	0.3 ± 0.1	3.0 ± 1.0	0.06 ± 0.02	0.06 ± 0.02

*Mean ± SEM. Shown are the number of colonies per 5×10^4 cells for both the treated and untreated bone marrow samples. N = 40.

between our current data and the lack of correlation of CFU-C (CFU-GM) with hematologic reconstitution previously reported from this center⁷ results from the different progenitor cell assays used. The culture conditions described in this report have a lower threshold for detection of progenitor cells following the 4-HC incubation (Table 1), and cultures of the grafts in this series using the previously reported assay techniques again demonstrated almost complete abrogation of clonal growth at this concentration of 4-HC. The two assay techniques differ greatly, and we cannot attribute the better growth in our current assay to any particular reagent(s). Because a threshold of detection also exists for the current assay, failure to grow only implies that the period of aplasia may be prolonged.

Many factors can affect the recovery of CFU-GM for bone marrow grafts manipulated ex vivo including incubation with 4-HC (as shown in this study) or other purging agents.^{22,23} Presumably the decreases in measurable (by in vitro culture) CFU-GM reflect a similar effect upon the primitive hematopoietic stem cell responsible for reconstitution following bone marrow transplantation. Other purging regimens, especially those that use monoclonal antibodies (MoAbs), which theoretically have the potential of distinguishing between committed and pluripotent progenitor cells, may dissociate the close relationship of CFU-GM to the hematopoietic stem cell implied by our results. The development of pluripotent stem cell assays^{11,24-27} may simplify the investigation of this aspect of BMT.

Although it is conceivable that the granulocyte colonies we observed arose from pluripotent stem cells, we would in this circumstance have expected to identify other colony types (erythroid, mixed granulo-erythroid) in the culture dishes as well. The stronger correlation of graft CFU-GM content to granulocyte rather than platelet or reticulocyte recovery may reflect the lineage commitment of this progenitor cell. How-

ever, other factors may have contributed to the relatively weaker correlation of the CFU-GM content with platelet and reticulocyte recovery. We routinely transfused platelets prophylactically for platelet counts below 20,000/ μ L, and for medical complications such as severe liver disease,¹³ CMV infection,¹⁴ and immune destruction,¹² may affect platelet survival or production. Similar considerations may affect the recovery of red cell production in these patients. In this series three patients died while still requiring platelet support after platelet recovery was predicted, but all had developed complications of transplantation prior to expected platelet recovery. Two patients who relapsed with acute myelogenous leukemia had prolonged platelet requirements without other explanation. The linear relationship of CFU-GM content to hematologic recovery and the occasional prolonged aplasias observed in this group of patients suggest that the preparative regimens employed are marrow-ablative and that spontaneous recovery is not responsible for the hematopoietic reconstitution of these patients.

These data confirm that the in vitro quantitation of CFU-GM can correlate with engraftment following autologous BMT with manipulated bone marrow grafts. Although other purging regimens may not demonstrate the same relationship, attention should be given to in vitro models of engraftment. The development of assays for more primitive pluripotent hematopoietic stem cells may simplify this task.

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Immunologic Aberrations, HIV Seropositivity and Seroconversion Rates in Patients With Hemophilia B

By Doreen B. Brettler, Frank Brewster, Peter H. Levine, Ann Forsberg, Sharon Baker, and John L. Sullivan

Because there have been reports that factor IX concentrate is less immunosuppressive and therefore factor IX users have less immunologic aberrations, we have studied a group of 22 patients with hemophilia B and six patients with factor VIII deficiency and high titer inhibitors with respect to lymphocyte numbers and function, human immunodeficiency virus (HIV) serology, and factor usage. This group was compared to 111 patients with hemophilia A and a group of 28 healthy male volunteer controls. When the study began in 1983, the majority of patients with hemophilia B and with higher titer factor VIII inhibitors were seronegative, 77% and 83% respectively, as compared to only 30% of patients with hemophilia A. At that time the factor IX users also had milder immune aberra-

tions than the hemophilia A group. However, with time and increasing clotting factor concentrate usage, seroconversion and more striking abnormalities in immune function have occurred in the hemophilia B group. In a subgroup of 16 patients with hemophilia B studied twice, the incidence of seropositivity increased from 31% in 1983 to 69% in 1985. We thus conclude that factor IX concentrate in itself is not less immunosuppressive than factor VIII concentrate. Seroconversion in factor IX concentrate users appears to be lagging behind seroconversion in factor VIII concentrate users, perhaps secondary to the lower cumulative dosage of concentrate that patients with hemophilia B utilize.

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THERE HAVE BEEN numerous reports of laboratory immune abnormalities, including reduced helper/suppressor lymphocyte ratios, anergy to cutaneously applied antigens, and decreased mitogen responsiveness in patients with factor VIII deficiency (hemophilia A).¹⁻⁴ The cause of these abnormalities is multifactorial, with the most important variables being the amount of factor concentrate infused and seropositivity to HIV (human immunodeficiency virus).⁴ Beginning in 1983 there have been suggestions in the literature that those patients with factor IX deficiency (hemophilia B) as well as those with high titer inhibitors to factor VIII receiving large amounts of factor IX concentrate have a lesser degree of immune dysfunction than patients with hemophilia A.⁵⁻⁹ However, in each of these studies the sample size was small, and correlations between seropositivity to HIV and immune data were not always available. In these studies it was implied that the factor IX concentrate was in itself less immunosuppressive. However, other reports suggested that immune aberrations were dependent on intensity of treatment. In one study, for example, patients who were intensively treated with factor IX concentrates were found to have decreased helper/suppressor cell ratios when compared to patients with hemophilia B who used minimal amounts of clotting factor concentrates.¹⁰

Patients with hemophilia B characteristically infuse fewer units per year than patients with hemophilia A. It remains

unclear, therefore, whether factor IX concentrate is less immunosuppressive than factor VIII concentrate or if the degree of immune aberration is directly related to the amount of total units infused. We have studied a group of patients with factor IX deficiency and also a group of factor IX concentrate recipients with high titer inhibitors to factor VIII with respect to lymphocyte number and function, HIV seropositivity, and factor usage. Additionally, to test the hypothesis that factor IX-deficient patients are lagging behind in HIV seroconversion and the development of immune defects because of lower cumulative factor doses, we restudied the HIV seroconverters again after two to three years for progressive alterations in immunoregulation.

MATERIALS AND METHODS

Patient selection. Twenty-two patients with hemophilia B who were enrolled at the New England Area Comprehensive Hemophilia Center were consecutively tested at the time of their routine annual visit. All were tested at least once in 1983/1984, and 16 were tested subsequently after a two-to-three-year period from 1983 to 1985. Fourteen patients had severe hemophilia B (factor level <1%), one patient had severe hemophilia B with a high titer inhibitor, and six had moderate hemophilia B (factor IX = 2% to 5%). A final patient had severe hemophilia B by laboratory measurement but was mild clinically and never had infused any blood products. All patients were on a home therapy program as described previously,¹¹ utilizing commercial factor IX concentrate from a variety of manufacturers except for the one patient who never infused concentrate and had last been infused with plasma approximately 30 years ago. All patients began to use heat-treated factor IX in January 1985. None of the 22 patients had acquired immune deficiency syndrome (AIDS) or AIDS related complex (ARC). Six of the 22 patients were noted to have small but palpable lymph nodes in the anterior and posterior cervical node chains. One patient had a six-week episode of weight loss and fevers that have subsequently resolved. In retrospect, this particular episode was most likely related to seroconversion to HIV positivity. The mean age of the group with hemophilia B was 25.7 years, with a range of 5 to 60 years.

Additionally, six patients with severe hemophilia A and high titer inhibitors, five of whom had received only factor IX products over the last five years and one who had received factor VIII concentrate in addition to factor IX concentrates, were studied. Their inhibitor titers ranged from 3.3 to 11.5 Bethesda Units. These patients ranged in age from 6 to 37 years, with a mean of 19 years. No patient in this

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Table 1. Characteristics of Each Group

	Mean Age (yr) (range)	Factor Usage U/kg/yr	Severity of Hemophilia
Normal controls n = 28	33.5 ± 10.2 (18-54)	N/A	N/A
Hemophilia A n = 111	23.9 ± 16.0 (2-72)	1384 ± 1253 Factor VIII	Mild 10 Moderate 9 Severe 92
Hemophilia B n = 22	25.7 ± 14.4 (5-60)	756 ± 910 Factor IX	Mild 0 Moderate 6 Severe 16
Factor VIII inhibitor patients n = 6	19.0 ± 10.5 (6-37)	5315 ± 3436 Factor IX	Severe 6

group had AIDS, ARC, lymphadenopathy, or thrombocytopenia. A group of 111 patients with hemophilia A that had been previously completely characterized with respect to immune function and HIV serology served as a comparison group.⁴ The nonhemophilic control group consisted of 28 healthy male volunteers on no medications who had a mean age of 33.5 years, with a range of 18 to 54 years. The characteristics of these groups are seen in Table 1.

Lymphocyte separation. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized venous blood by centrifugation on Ficoll-Hypaque (FH; Pharmacia Fine Chemicals, Inc, Piscataway, NJ) density gradients. PBMC preparations contained 70% to 80% lymphocytes, 10% to 20% monocytes, and 20% polymorphonuclear leukocytes as determined by cell morphology. All studies were performed on PBMC maintained at 37°C overnight in HEPES-buffered RPMI 1640 medium (Grand Island Biological Co, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), and 25 µg/mL gentamicin (Grand Island Biological Co, Grand Island, NY).

Lymphocyte surface marker studies. Relative percentages of T lymphocyte populations were enumerated using indirect immunofluorescence and the following reagents: mouse monoclonal antibodies (MoAbs) OKT.11 (pan-T cell), OKT.4 (T helper/inducer) and OKT.8 (cytotoxic/suppressor; Ortho Pharmaceutical Co, Raritan, NJ). HLA-DR-bearing lymphocytes were enumerated using direct immunofluorescence with a fluorescein-conjugated mouse MoAb to a common determinant of human HLA-DR from Becton Dickinson Company (Mountain View, CA). All samples were analyzed with the use of a Becton Dickinson FACS IV. Absolute numbers of the various cell population per microliter of whole blood were calculated by multiplying their relative percentage by the absolute numbers of

peripheral blood mononuclear cells as determined from complete blood counts.

Lymphocyte proliferation studies. Using a standard microculture method,¹² 5×10^4 PBMC in 0.25 mL of RPMI 1640 with 10% fetal calf serum (FCS) were distributed into replicate wells of Microtiter II plates (Falcon Labware, Division of Becton Dickinson and Co, Oxnard, CA). Mitogens were added in the following concentrations: phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC) 0.2 to 8.0 µg/mL; pokeweed mitogen (PWM; PL Biochemicals, St. Louis) 0.05 to 5 µg/mL; concanavalin A (Con A; Calbiochem-Behring Corp, American Hoechst Corp, San Diego, CA) 0.4 to 40 µg/mL. Cultures were terminated on day 3 for PHA, day 5 for Con A and PWM. All cells were cultured in a 5% CO₂ environment at 37°C. Eighteen hours before harvesting, the cultures were pulsed with 2 µCi of ³H-thymidine, (³HTdR; sp act 6.7 mCi/mmol/L; New England Nuclear, Boston). Cells were harvested onto glass-fiber filters, and ³H-thymidine content was determined by scintillation counting. All cultures were performed in triplicate.

Delayed cutaneous hypersensitivity. Delayed cutaneous hypersensitivity (DCH) was measured in both hemophiliacs and controls using the Multitest CMI (Merieux Institute Inc, Miami) skin test set.¹³ Seven test recall antigens (tetanus toxoid, diphtheria toxoid, streptococcus, proteus, tuberculin, candida, and trichophyton) and a glycerine-vehicle control were simultaneously applied on the volar surface of the arm with the multipuncture set. The skin tests were read at 48 hours, and the presence of induration of greater than 2 mm for each antigen was recorded. A patient was considered anergic if no skin tests were positive after 48 hours and a responder if one or more skin tests were positive.

HIV serology. Serum samples were assayed for IgG antibody to HIV using a modification of an indirect immunofluorescent assay described previously.¹⁴ Patient sera were also analyzed for antibody to HIV viral proteins by the Western blot method.¹⁵ Briefly, sodium dodecyl sulfate (SDS)-disrupted HIV (kindly provided by R.C. Gallo) was fractionated on a discontinuous polyacrylamide-SDS gel system and then electrophoretically transferred to a sheet of nitrocellulose (Schleicher and Schuell, Keene, NH) by methods described previously.¹⁶ After blocking with 5% nonfat dry milk saline solution, the Western blot was then cut into strips and reacted with a 1:100 dilution of patient serum overnight at room temperature. The strips were first reacted with biotinylated goat antihuman IgG and then avidin D-conjugated horseradish peroxidase (Vector Labs, Burlingame, CA). A 4-chloro-1-naphthol substrate solution was added for ten minutes, then the color reaction was stopped and the banding pattern compared to patterns obtained with HIV positive sera, with

Table 2. Lymphocyte Markers in Each Group at Outset of Study

	Normal Controls (n = 28)	Hemophilia A Patients (n = 111)	Hemophilia B Patients (n = 22)	Inhibitor Patients (n = 6)
Helper cells				
Absolute cells/µL	965 ± 321	789 ± 560*	851 ± 384	856 ± 398
Percent	48.1 ± 8.3	32.4 ± 11.6†	36.7 ± 7.9*‡	38.4 ± 12.6
Suppressor cells				
Absolute cells/µL	513 ± 195	722 ± 868†	760 ± 285†‡	531 ± 181
Percent	25.9 ± 7.3	37.1 ± 10.6†	34.1 ± 11.8*‡	25.4 ± 6.4
Helper/suppressor ratio	2.0 ± 0.7	1.0 ± 0.6†	1.3 ± 0.7†	1.7 ± 1.0
HLA-DR positive cells (%)	23.6 ± 3.0	30.4 ± 1.1*	23.7 ± 2.2‡	—

Results expressed as mean ± SD.

* $P < 0.025$ by Student's *t* test when compared to control.

† $P < 0.005$ when compared to control.

‡ $P < 0.025$ when compared to patients with hemophilia A.

Table 3. Mitogen Responses in Each Group at the Outset of the Study (1983)

	Normal Controls (n = 28)	Hemophilia A (n = 111)	Hemophilia B (n = 22)
Phytohemagglutinin (PHA)	80,740 ± 6,489	46,028 ± 2,999*	46,386 ± 6,333*
Concanavalin A (Con A)	130,194 ± 10,481	50,808 ± 4,247*	56,598 ± 7,411*
Pokeweed mitogen (PWM)	46,079 ± 5,212	20,404 ± 1,697*	22,697 ± 3,158*

* $P < 0.005$ when compared to controls. All results are expressed as mean CPM ± SEM.

special note made of antibody reactivity to p24 (viral core antigen) and gp41 (envelope glycoprotein).

Statistical analysis. Statistical differences between groups were evaluated by the use of Student's *t* test.

RESULTS

Surface markers. As can be seen in Table 2, patients with hemophilia B had significantly decreased percentages of T helper cells ($36.7 \pm 7.9\%$ v $48.1 \pm 8.3\%$, $P < 0.005$) when compared to the nonhemophilic control group. The absolute number and the percentages of suppressor cells were increased and the helper/suppressor ratio significantly decreased when compared to controls. When compared to the group with hemophilia A, however, absolute number and percentage of helper cells were increased, and relative amounts of suppressor cells were significantly decreased, making the helper/suppressor ratio higher and more similar to the normal controls. Percentages of HLA-DR cells were also not significantly different from the control population. The six patients with factor VIII deficiency and high titer inhibitors, although utilizing large amounts of factor IX, had in vitro immunologic tests that were not significantly different from the normal controls.

Mitogens. The responses to the mitogens PHA, Con A, and PWM were all significantly decreased in the patients with hemophilia B when compared to controls, as can be seen in Table 3. The responses were similar to those patients with hemophilia A.

Delayed cutaneous hypersensitivity. As we have reported previously,¹⁷ no patient with hemophilia B was anergic, as compared to 51% of patients with hemophilia A. Of the 17 patients on whom results were available, all had at least one skin test that was positive. The mean number of tests that were positive for hemophilia B patients was 2.5 ± 1.2 as compared to 1.2 ± 0.1 for hemophilia A patients and 3.5 ± 1.5 for controls (data not shown).

Factor concentrate usage. The group of patients with hemophilia B used significantly less clotting factor concentrate than those with factor VIII deficiency (757 ± 910 units factor IX/kg/yr v 1384 ± 252 units factor VIII/kg/yr, $P < 0.005$). Interestingly, those with inhibitors to factor VIII, although normal from an immunologic standpoint, used significantly increased amounts of factor concentrate when compared to both those with factor IX deficiency (5315 ± 3436 units factor IX/kg/yr v 758 ± 909 units factor IX/kg/yr, $P < 0.005$) and those with factor VIII deficiency (5315 ± 3435 units factor IX/kg/yr v 1384 ± 1252 units factor VIII/kg/yr, $P < 0.005$).

HIV serology. When comparing serology among the different groups, it is notable that in 1983, the first year that the patients were studied, 17/22 or 77% of patients with hemophilia B were seronegative as well as 5/6 patients with inhibitors to factor VIII. This is compared to only 30% of patients with factor VIII deficiency who were initially seronegative. The five patients with hemophilia B who were initially seropositive did use significantly higher amounts of factor IX concentrate than those with hemophilia B who were seronegative (Table 4). Thus it appears that in the hemophilia B population, increased amounts of clotting factor concentrate usage increases the incidence of seropositivity. In contrast to what has been reported in the group with hemophilia A,⁴ seropositivity to HIV in the group with hemophilia B studied in 1983 did not correlate with either decreased responsiveness to PWM or an increased amount of circulating HLA-DR-positive lymphocytes (Table 4).

In the patients with inhibitors to factor VIII who use large amounts of factor IX, the initial seropositivity rate was only 1/6 or 16.6%. It remains unclear why the patients who utilized the most factor IX, the inhibitor patients, had an initial low rate of seropositivity; this may only be a reflection of the small sample size for this group.

Longitudinal studies. Sixteen patients with hemophilia B were studied in year 1 (1983) and then in a subsequent

Table 4. Variables Correlating With HIV Seropositivity in 1983

	Hemophilia A: HIV Serology		Hemophilia B: HIV Serology	
	Negative (n = 28)	Positive (n = 83)	Negative (n = 17)	Positive (n = 5)
Factor usage				
U/kg/yr	1,033 ± 339	1,732 ± 150*	424 ± 396	1,710 ± 1,294†
Response to PWM (maximum cpm)	29,752 ± 4,847	17,413 ± 1,482*	21,334 ± 4,117	23,485 ± 6,464
% HLA-DR				
Positive Cells	24.0 ± 1.9	32.7 ± 1.3*	24.5 ± 2.8	23.0 ± 4.4

All results expressed as mean ± SD except for response to PWM expressed as mean ± SEM.

* $P < 0.001$ when compared to seronegative counterpart.

† $P < 0.005$ when compared to seronegative counterpart.

year, year 2 (1984 and/or 1985) with respect to immune function and HIV serology. In this subgroup of patients the number of those who were seropositive increased from 5/16 (31%) in 1983 to 11/16 (69%) in 1985. There were six seroconverters during that time period. When these six patients were compared with respect to immunologic parameters between year 1 and year 2, there was a decrease in T helper/suppressor ratio from 1.65 ± 0.95 to 1.15 ± 0.50 , which did not reach significance (Fig 1), and a trend toward a decrease in absolute T helper cells and an increase in absolute T suppressor cells (Fig 2). One patient who seroconverted did have an acute but prolonged febrile illness in retrospect associated with seroconversion. Otherwise the group of seroconverters did not differ clinically from the group as a whole.

The five patients who remained seronegative actually had an increase in the helper/suppressor cell ratio from 1.28 ± 0.69 to 1.46 ± 0.76 secondary to a slight increase in helper cells (data not shown). They also used clotting factor concentrates from a variety of manufacturers and did not differ clinically from the patients who seroconverted. Interestingly, it is of note that the utilization of factor concentrate over the study period 1983 to 1985 was not significantly different between the patients who seroconverted and the patients who remained seronegative (330 ± 323 units/kg/yr v 706 ± 497 μ /kg, P is not significant). However, these subsets represent very small patient groups, and thus statistical comparison is difficult.

The five patients who were initially seropositive remained the same immunologically with decreased helper/suppressor ratios secondary to both decreased helper cells and increased suppressor cells. It should be noted that the intensity of treatment had not changed in these patients during the interval between the two sets of studies.

The six patients with inhibitors were also studied longitudinally. Three patients seroconverted. The mean helper/suppressor cell ratio of the six patients at the outset of the study was 1.70 ± 0.96 , whereas in 1985, after the three seroconversions occurred, the mean ratio was 0.77 ± 0.26 , a significant downward trend ($P < 0.025$).

DISCUSSION

In numerous reports, patients with hemophilia B have been shown to have less immunologic aberrations than

Fig 1. Decrease in the T helper cell/T suppressor cell ratio in the patients with hemophilia B who seroconverted during the study period. I represents the first year of study and II the subsequent study year. The difference in the mean ratio between the two groups is not significant. Each dot represents an individual patient.

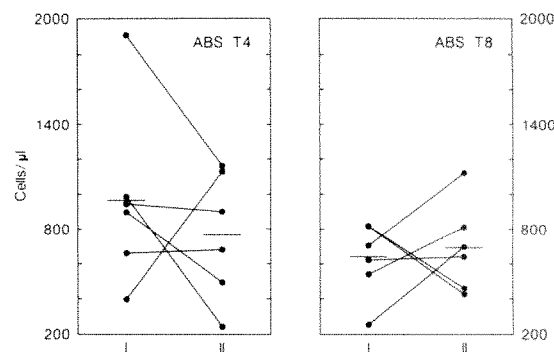
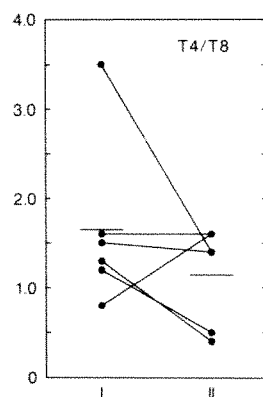


Fig 2. Absolute numbers of T helper cells and T suppressor cells studied in the seroconverters over time. I represents the first year of study when patients were seronegative and II the subsequent study year when they had seroconverted. The trend shows a decrease in the mean absolute T helper cells and an increase in mean absolute T suppressor, but the differences are not statistically significant.

patients with factor VIII deficiency. It has been found that the factor IX concentrates have decreased amounts of beta-2 microglobulin protein when compared to factor VIII concentrates. This has been postulated as a reason why these concentrates might cause less immunosuppression than do factor VIII concentrates.⁸ Recently it has been reported that a prolonged exposure to ethanol during plasma fractionation can inactivate HIV virus.¹⁸ Since certain factor IX products, especially activated prothrombin complex used intermittently by patients with inhibitors, are made utilizing such a step, less immune aberrations have been postulated to occur in patients using these products. In one study, patients treated only with activated prothrombin complex were shown to have no seroconversion over a three-year period.¹⁹ Another reason put forth that patients with hemophilia B may have more normal immune function is that a smaller percentage of patients with hemophilia B are HIV antibody positive. In Great Britain only 6.3% of 316 hemophilia B patients studied were seropositive.²⁰

In our group of 22 patients with hemophilia B and six patients with inhibitor to factor VIII studied initially in 1983, immunologic abnormalities were less striking than in a comparison group of patients with hemophilia A and more similar to a normal control group. When compared to patients with hemophilia A, we found, as previously reported by others,^{6,9} that helper/suppressor ratios were higher as a result of increased amounts of helper cells and decreased numbers of suppressor cells. Skin test anergy was also absent in all patients with factor IX deficiency and also the inhibitor patients.

In 1983 the seropositivity rate in the patients with hemophilia A was 72%, in contrast to a rate of 31% in patients with hemophilia B and 16.6% in patients with high titer inhibitors. Seropositivity to HIV was correlated with higher clotting factor concentrate usage in the population with hemophilia B found to be seropositive at first testing. This finding is comparable to that of Mannucci et al²¹ as well as to data from other European and American hemophilia centers.^{22,23} Overall, the amount of factor infused was less than in the

population with factor VIII deficiency. Based on the above information, one could hypothesize that the factor IX itself is not less immunosuppressive but that less of it was used per patient. Seropositivity and subsequent immunologic aberrations should emerge later, in time, as either the cumulative dose increases or the probability of exposure to an HIV-contaminated lot increases.

Supporting this hypothesis is the data on the 16 patients with hemophilia B who were studied over at least two consecutive years. There was a 38% seroconversion rate, with 6/16 becoming seropositive. Correlated with seroconversion was a trend toward worsening of immune function. The incidence of seropositivity in the hemophilia B population may never reach that in patients with hemophilia A, since future seroconversion may well be prevented by the newer heat-treated concentrates that will likely eliminate further HIV exposure.

Thus it appears that factor IX concentrate itself is not less immunosuppressive but that less per year has been utilized in the hemophilia B patients when compared to factor VIII usage in hemophilia A patients. The correlation between amount of factor infused, conversion to HIV antibody positivity, and subsequent immunologic aberrations when examined in 1983 is similar to that seen in patients with factor

VIII deficiency, only delayed. It is of note that the patients who seroconverted during the study years did not use more factor concentrate over the three-year span than those who continued to remain HIV antibody negative. Thus not only cumulative dose but chance exposure to a HIV-contaminated lot could also be important. These results also suggest that a more purified factor IX preparation is needed to decrease the immunosuppression observed in patients with hemophilia B receiving concentrate infusions.

The data concerning the factor VIII inhibitor patients are difficult to interpret, since the group represents a small subset. Because the patients appeared at the outset of the study to have normal immune studies, they may have been, in some manner, protected from the immune suppressant effects of large doses of factor IX concentrate. However, over the course of two years, 3/6 patients with inhibitors seroconverted, and their immune function deteriorated. This is consistent with the immunologic deterioration seen in patients with hemophilia A or B following HIV infection.

ADDENDUM

Since this paper was originally submitted, 21 of the 22 original patients with hemophilia B have seroconverted as well as five of the six inhibitor patients.

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Plasma and Platelet HLA in Normal Individuals: Quantitation by Competitive Enzyme-Linked Immunoassay

By K. J. Kao

Recent studies on platelet HLA indicate that >50% of platelet HLA antigens are adsorbed on the platelet surface and may be derived from plasma. It has been speculated that platelet HLA may be directly proportional to plasma HLA concentration. To determine the quantitative correlation between plasma and platelet HLA, a precise competitive enzyme-linked immunoassay (ELISA) for measurements of soluble and cellular HLA antigens was developed by using purified HLA antigens and W6/32 anti-HLA monoclonal antibody. The useful range of the standard curve for the assay was 0.01 to 5.0 $\mu\text{g/mL}$. The intraassay and interassay variations were 7% and 14%, respectively. The plasma HLA concentrations measured in 61 healthy adults ranged from 0.25 to 4.1 $\mu\text{g/mL}$, and the mean plasma HLA concentration was $1.47 \pm 0.87 \mu\text{g/mL}$ ($\pm\text{SD}$). Platelet HLA concentrations determined in the same 61 persons ranged

from 4.7 to 17.33 fg/platelet, and the mean concentration was $9.3 \pm 2.9 \text{ fg/platelet}$ ($\pm\text{SD}$). Chloroquine-elutable platelet HLA concentrations were also determined in 42 of the 61 persons, with the mean value of $5.7 \pm 2.1 \text{ fg/platelet}$ ($\pm\text{SD}$). The plasma HLA concentration of each individual was then correlated with the same person's total or chloroquine-elutable platelet HLA concentration. Linear regression analyses of the results revealed no significant correlation between platelet and plasma HLA concentrations. Thus, it is unlikely that chloroquine-elutable HLAs are derived from plasma. The developed solid-phase assay for HLA will be useful for further study of the quantitative significance of plasma HLA antigens in allosensitization of transfused individuals.

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CLASS I MOLECULES of the human major histocompatibility complex (HLA) are the most important antigenic system in determining the survival of transfused platelets in alloimmunized recipients.¹⁻³ Recently, on the average of 50,000 to 120,000 molecules of HLA antigens per platelet were demonstrated to exist among different individuals,⁴ and >50% of platelet HLA antigens can be selectively removed by acid-hypertonic chloroquine without affecting platelet membrane integrity⁵ and other platelet membrane glycoproteins.^{5,6} The platelet-surface HLA antigens removed by chloroquine could also be detected in the eluate.^{4,5} These results suggest that most platelet HLA antigens are adsorbed on the platelet surface and do not span the platelet plasma membrane.

The existence of soluble HLA antigens in serum^{7,8} was first reported in 1970. Subsequent studies on a few individuals suggested that quantitative expression of HLA-B12 antigen on platelets was proportional to the serum content of that antigen.⁹ Later, Lalezari and Driscoll¹⁰ qualitatively demonstrated that platelets have the ability to acquire HLA specificity from plasma. These results suggest that adsorbed platelet HLA antigens may be derived from plasma and that platelet HLA antigens may be quantitatively proportional to plasma HLA antigens. In addition, Pellegrino and associates¹¹ reported that anti-HLA antibodies were elicited in individuals who had received intravenous (IV) infusion of

human plasma. Their finding indicates that plasma HLA is immunogenic. Nevertheless, concentrations of soluble plasma HLA antigen have only been measured semiquantitatively by inhibition of a lymphocytotoxicity test^{12,13} or by neutralization of a platelet cytotoxicity test.^{9,14}

We report the development of a competitive enzyme-linked immunoassay (ELISA) for precise quantitation of both plasma soluble and platelet cellular HLA antigens. The assay uses purified HLA antigens and W6/32 anti-HLA monoclonal antibody, and was applied to study the quantitative correlation between platelet and plasma HLA concentrations. The potential application of the assay to study plasma HLA immunogenicity is discussed.

MATERIALS AND METHODS

Isolation of W6/32 anti-HLA monoclonal antibody. The W6/32 hybridoma, obtained from the American Type Culture Collection (Rockville, MD), was originally developed by Barnstable, and co-workers¹⁵ and produces an IgG_{2b} monoclonal antibody that recognizes a common monomorphic determinant on HLA-A, B, and C molecules. W6/32 anti-HLA monoclonal antibody (W6/32 McAb) was isolated from culture medium by protein A-affinity column chromatography as described previously.⁴

Purification of HLA antigens. Sixty units of outdated platelet concentrates were obtained from the Civitan Regional Blood Center (Gainesville, FL). Platelets from each unit of platelet concentrate were washed twice with 50 mL of 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.15 mol/L of NaCl and 2 mmol/L of EDTA (Tris-saline-EDTA). Washed platelets were pooled, centrifuged at 10,000 g for 10 minutes, and solubilized by resuspension in a equal volume of 2% NP-40 made in 0.02 mol/L of Tris-HCl, pH 7.4, containing 1 mmol/L of phenyl-methyl-sulfonyl fluoride (PMSF) and 1 mmol/L of ϵ -amino-*n*-caproic acid (EACA). The resuspended platelets were incubated on ice for 60 minutes. Solubilized platelets were then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was collected and centrifuged again at 50,000 g for 60 minutes at 4°C. Solubilized HLA antigens were then isolated from the final supernatant by column chromatography on the following three sequentially linked columns: (a) a Sepharose-4B column, (b) a normal mouse IgG-Sepharose-4B column, and (c) a W6/32 McAb-Sepharose 4B column. Details of preparing the columns have been described previously.¹⁶ The size of each column was 1.6 $\times$ 6 cm. After

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extensive washing, HLA antigens were eluted by 0.05 mol/L of diethylamine, pH 11.5, containing 0.1% NP-40 and collected in 1-mL fractions. The eluted protein peak was pooled and dialyzed extensively against 0.01 mol/L of phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Na₂S₂O₅ and 0.1% NP-40. The concentration of HLA antigens was determined by a modified Lowry assay¹⁷ using bovine serum albumin (BSA) as the standard. Homogeneity of purified HLA antigens was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).¹⁸

Preparation of platelet samples for HLA assay. Platelets were isolated from freshly drawn venous blood by differential centrifugation and washed as described.⁴ Routinely, the venous blood was drawn into a 7-mL vacutainer containing EDTA as an anticoagulant. The washed platelets were suspended in 3 mL of Tris-saline-EDTA buffer and enumerated by a S-plus Coulter counter (Coulter Electronics, Hialeah, FL). The contamination of WBCs is always <0.01%. Ten to 40 million washed platelets were pipetted into a 1-mL conical polystyrene tube and pelleted by a Beckman Microfuge B (Beckman Instruments, Palo Alto, CA) at room temperature for 2 minutes. The supernatant was aspirated and discarded. Platelets were then resuspended in 100 μ L 0.02 mol/L of Tris-HCl, pH 7.4, containing 0.05% NP-40, 5 mmol/L of EDTA and 1 mmol/L of PMSF, and left on ice for 60 minutes with occasional vortexing. After solubilization, all samples were stored at -70°C until assayed. The frozen samples were stable for at least 12 months at -70°C. Chloroquine-treated platelets were prepared as reported previously.⁴

Competitive ELISA for HLA antigens. Each well of a 96-well Immunoplate (Nunc Inter-Med, Kamstrup, Denmark) was incubated overnight with 100 μ L of purified HLA antigens (2 μ g/mL) diluted in 0.01 mol/L of sodium phosphate buffer, pH 7.2, containing 0.15 mol/L of NaCl and 0.02% NaN₃ (PBS-azide) at 4°C. Subsequently, each well was washed three times with PBS-azide containing 0.5% Tween-20 (PBS-T) and then blocked with 300 μ L 1% BSA in PBS-T for 30 minutes at room temperature. After the wells were washed three times, 50 μ L of different concentrations of purified HLA standard (0.01 to 5 μ g/mL), diluted plasma or solubilized platelet samples were added to wells in duplicate, followed by the addition of 50 μ L W6/32 anti-HLA McAb (0.02 μ g/mL). The incubations were mixed by placing plates on a Micro-Shaker II (Dynatech Laboratories, Alexandria, VA) for 15 seconds, and incubated at room temperature for 90 minutes. The plate was washed three times again, followed by addition of 100 μ L of rabbit antimouse IgG alkaline phosphatase conjugate (1,000 $\times$ dilution) (Sigma Chemical, St Louis) into each well and incubated for 30 minutes.

Finally, 200 μ L *p*-nitrophenyl phosphate disodium (1 mg/mL prepared in 0.01 mol/L of sodium bicarbonate buffer, pH 9.6, containing 2 mmol/L of MgCl₂) was added to each well, and the plates were incubated at room temperature for 90 to 120 minutes in the dark. Fifty microliters of 3N NaOH was then added to each incubation to stop the reaction. Absorbance of each well at a wave length of 405 nm was measured in a BioRad ELISA plate reader (BioRad Laboratories Richmond, CA). All samples were diluted as needed in PBS-T containing 1% BSA and 5 mmol/L of EDTA and were assayed in duplicate. Plasma samples were also assayed at two different dilutions (5 $\times$ and 10 $\times$) to ensure absence of interfering factors.

RESULTS

Isolation of platelet HLA antigens. HLA antigens isolated from solubilized platelets appeared to be homogeneous as judged by SDS-PAGE (Fig 1). The purified HLA antigens consist of a 44-kd heavy chain and a 12-kd noncoval-

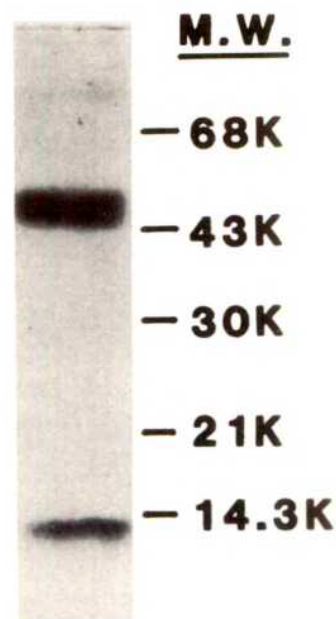


Fig 1. Sodiumdodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis of purified HLA antigens. The purified HLA antigens consist of a 44-kd heavy chain and a 12-kd noncovalently associated β_2 -microglobulin. The same two polypeptide chains were also noted after reduction by 5% mercaptoethanol.

ently associated β_2 -microglobulin. Routinely, 2 to 3 mg of HLA antigens were recovered from 60 U of outdated platelet concentrates. These purified HLA antigens were used to coat the wells and served as standards for the assay.

Competitive ELISA for HLA antigens. The quantitative assay for HLA antigens was developed based on the principle of competition for the binding of W6/32 anti-HLA McAb between plate-bound HLA antigens and unbound HLA

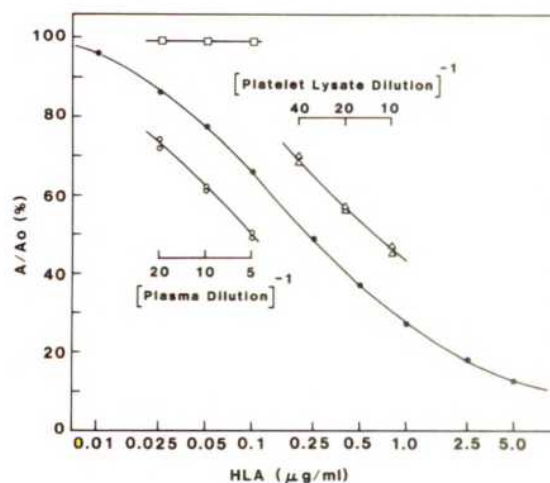


Fig 2. Standard curve of the competitive enzyme-linked immunoassay for HLA antigens (—●—). (A) absorbance of each well at 405nm; (Ao) mean absorbance of duplicated wells in which no purified HLA antigen was added. Open circles represent 5 $\times$, 10 $\times$, and 20 $\times$ dilutions of pooled normal plasma (—○—); squares, same dilutions of HLA-depleted pooled plasma (—□—). Dilutions of 4×10^7 platelets solubilized in 100 μ L of lysing buffer (—△—).

antigens in the standards or unknown samples. The concentrations of the HLA antigens used to coat the wells ($2.0 \mu\text{g/mL}$) and W6/32 anti-HLA McAb ($0.02 \mu\text{g/mL}$) used in the assay were found to be optimal to gain greater sensitivity and precision. As shown in Fig 2, the useful range of the standard curve was between 0.01 and $5.0 \mu\text{g/mL}$.

When plasma pooled from six different healthy adults without history of previous blood transfusion or pregnancy was assayed at different dilutions, the values were in parallel with the standard curve (Fig 2). The result indicates that there was no factor(s) in plasma interfering with the assay. Next, the specificity of the assay was evaluated by assaying a HLA-depleted pooled plasma prepared by incubating pooled plasma with equal volumes of PA2.6 anti-HLA McAb-Sepharose 4B beads at 4°C for 4 hours. PA2.6 anti-HLA antibody was an IgG₁ monoclonal antibody developed by Brodsky and Parham.¹⁹ As expected, no significant amount of HLA could be measured in the sample after absorption (Fig 2).

Next, both plasma and serum samples were prepared from each of six different volunteer donors and assayed for HLA concentrations. No significant difference was observed between plasma and serum HLA concentrations ($P > .5$). The intraassay and the interassay assay variations were evaluated according to the method described by Bauer and Kennedy.²⁰ Pooled normal plasma was divided into aliquots and frozen at -70°C . On each of the ten different dates, one aliquot was thawed and assayed in duplicate. The intraassay and interassay variations were 7% and 14%, respectively.

Quantitation of platelet HLA concentrations. To measure cellular HLA concentration with the developed competitive ELISA, washed platelets (4×10^7 cells) were solubilized with 0.5% NP-40. The solubilized platelet sample was serially diluted and assayed. As shown in Fig 2, the results

were in parallel with the standard curve of the assay, which excluded presence of any interfering factor(s) in the solubilized platelet samples.

To determine whether the amount of detergent used was sufficient to solubilize platelets, 1×10^7 to 4×10^7 washed platelets from each of three different individuals were lysed by $100 \mu\text{L}$ of solubilization buffer and then assayed. If $100 \mu\text{L}$ of 0.5% NP-40 is capable of solubilizing all the platelets and releasing all cellular HLA antigens, a linear correlation should be expected between HLA contents and the number of platelets solubilized. As shown in Fig 3, the amount of HLA indeed was linearly proportional to the number of platelets solubilized. Thus, $100 \mu\text{L}$ of 0.5% NP-40 was sufficient to release HLA from $\leq 4 \times 10^7$ platelets.

The competitive ELISA was further evaluated by comparing this method with the surface binding assay in which ^{125}I -labeled W6/32 Fab fragments were used. Concentrations of platelet HLA of four different individuals were determined by these two separate methods. As shown in Table 1, platelet HLA concentrations determined by either method yielded similar results.

Quantitative correlation between plasma and platelet HLA concentrations. After validation of the assay, plasma and platelet samples were collected from 61 healthy volunteer donors and assayed for their HLA concentrations. The range of plasma HLA concentrations was from 0.25 to $4.1 \mu\text{g/mL}$, and the mean plasma HLA concentration was $1.47 \pm 0.87 \mu\text{g/mL}$ (mean $\pm$ SD). Platelet HLA concentrations of the same 61 persons ranged from 4.7 to $17.33 \text{ fg/platelet}$, and the mean concentration was $9.3 \pm 2.9 \text{ fg/platelet}$ (mean $\pm$ SD). The plasma HLA concentration of each individual was then correlated with his or her own platelet HLA concentration. Linear regression analysis²¹ showed no significant correlation between the two parameters ($r = .06$ and $P > 0.5$) (Fig 4).

HLA concentrations of chloroquine-treated platelets were also determined in 42 of the 61 volunteer donors. The quantities of chloroquine-elutable platelet HLA antigens were then calculated as the difference between HLA concentration of platelets before and after the treatment of chloroquine. The range of chloroquine-elutable platelet HLA antigens among the 42 individuals was 2.5 to 10.3 fg/platelet . The mean chloroquine-elutable platelet HLA was 5.7 ± 2.1

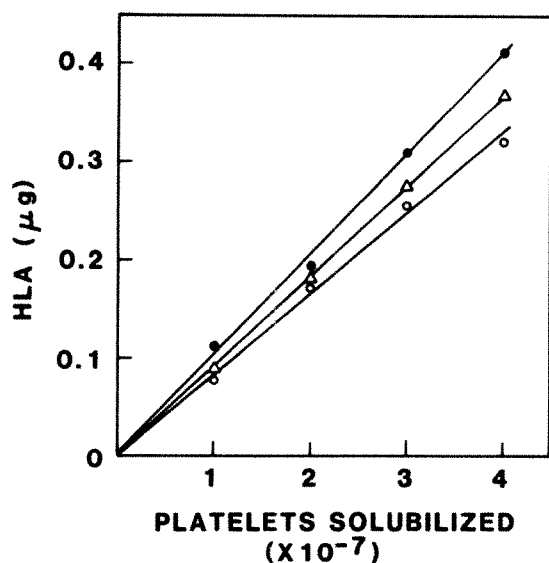


Fig 3. HLA contents of solubilized platelets. Different numbers of platelets were solubilized with $100 \mu\text{L}$ of lysing buffer. Amount of HLA antigen was measured by the enzyme-linked immunoassay. Each line represents platelets prepared from one individual.

Table 1. Platelet HLA Concentrations Determined by EIA and SBA

Case No.	Platelet HLA Concentration (fg/platelet)	
	EIA	SBA*
1	11.5	10.7
2	7.7	8.1
3	7.9	8.6
4	6.7	6.5

EIA, enzyme-linked immunoassay; SBA, surface binding assay.

*SBA was performed by using ^{125}I -labeled W6/32 monoclonal Fab fragments and Scatchard analysis.⁴ The concentrations of platelets HLA were converted from molecules per platelet to femtograms per platelet. The mol wt of 56 kd for HLA antigens was used for calculation. There was no significant difference between HLA concentrations determined by the two assays ($P > .5$).

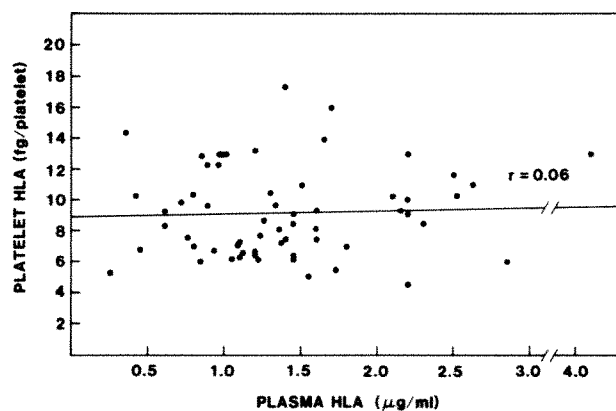


Fig 4. Quantitative correlation between plasma and total platelet HLA concentrations. Sixty-one normal adults were included in the study. Linear regression analysis revealed no significant correlation between the two parameters ($r = .06$, $P > .5$).

fg/platelet (mean $\pm$ SD). Again, no significant correlation was observed between chloroquine-elutable platelet HLA and the same person's plasma HLA concentration ($r = .03$ and $P > .8$). (Fig 5).

DISCUSSION

Although quantitation of plasma and platelet HLA antigens has been reported,^{9,12-14} these previous studies used inhibition of lymphocytotoxicity or platelet cytotoxicity as tests to measure relative rather than exact quantities of the HLA antigens. Theoretically, the degree of inhibition in those cytotoxicity assays is proportional to the actual quantity of HLA antigens. The HLA antigen concentrations determined can be affected, however, by microheterogeneity of the same serologically defined HLA antigen, by presence of undefined HLA antigens, and by cross-reactivity between different specific HLA antigens. Furthermore, the inhibition

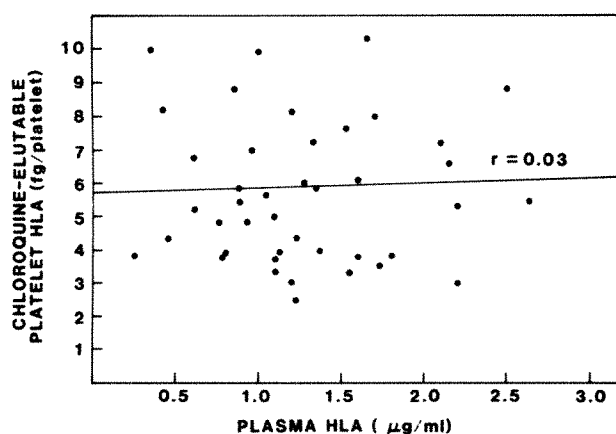


Fig 5. Quantitative correlation between plasma and chloroquine-elutable platelet HLA concentrations. Chloroquine-elutable platelet HLA was determined as the difference of HLA concentration between platelets before and after chloroquine treatment. Forty-two subjects were available for the study. Linear regression analysis did not reveal any significant correlation between the two parameters ($r = .03$, $P > .8$).

of cytotoxicity assay measured only concentration of one specific type of HLA antigens at one time.

We describe competitive EIA for precise quantitation of total plasma soluble and platelet cellular HLA antigens. As shown in Table 1, the concentrations of platelet cellular HLA antigens that were determined by the competitive EIA were comparable with those determined by the surface binding assay using I^{125} -labelled Fab fragments of W6/32 anti-HLA McAb and Scatchard analysis. The results indicate that all the platelet HLA antigens measured by the competitive EIA were on the platelet surface, and there was no significant amount of HLA antigens in platelet cytoplasm. This finding is consistent with the absence of active protein synthesis in platelets. In contrast, the concentration of lymphocyte HLA antigens measured by the described competitive ELISA, was two to three times higher than that measured by the surface binding assay (data not shown). These differences are most likely due to the release of cytoplasmic HLA antigens from lymphocytes during solubilization. Thus, the HLA antigens measured in nucleated cells by the ELISA represent total cellular HLA antigens, which includes cytoplasmic and surface HLA antigens. The active synthesis of HLA antigens and the relatively fast turnover of membrane HLA antigens in lymphocytes²² are most likely responsible for the presence of high levels of HLA antigens in cytoplasm.

To test if the presence of anti-HLA antibodies in plasma samples from individuals with previous pregnancies or multiple blood transfusions might interfere with the assay, five of such plasma samples were assayed at $5\times$, $10\times$, and $20\times$ dilutions. Due to the presence of anti-HLA antibodies that interfered with the assay, the values obtained were not in parallel with the standard curve of the assay (data not shown). Therefore, to obtain a valid measurement of plasma or serum HLA concentration, it is imperative to assay all plasma samples at least at two different dilutions to exclude those samples containing interfering factor(s). All the plasma HLA concentrations assayed in this report were measured at $5\times$ and $10\times$ dilutions. The results were all in parallel with the standard curve.

According to the present study, the average plasma HLA concentration in normal adults is $1.47 \mu\text{g/mL}$. This number indicates that a significant amount of HLA antigens is present in the plasma of various blood components. Although the potential immunogenicity of plasma HLA antigens was reported,¹¹ the clinical significance of the quantitative variation of plasma HLA in allosensitization of recipients of plasma containing blood products remains unclear. The precise quantitation of plasma HLA antigens by using the developed assay will facilitate the study of plasma HLA immunogenicity.

Because previously platelet HLA-B12 antigen concentrations was shown to be proportional to plasma HLA-B12 content,⁹ that platelets can acquire plasma HLA antigens¹⁰ and that most platelet HLA is probably adsorbed on platelet surface,^{4,5} it has been speculated that the amounts of platelet-surface HLA would be directly proportional to plasma HLA levels. Results of the present study showed that there was no significant quantitative correlation between total platelet

and plasma HLA (Fig 4). Because it is possible that only the adsorbed platelet HLA may derive from plasma and may correlate to plasma HLA, quantities of adsorbed platelet HLA were then determined in 42 individuals by subtracting HLA concentration left on chloroquine-treated platelets from total platelet HLA content. Nevertheless, no quantitative correlation between plasma and adsorbed platelet HLA was observed (Fig 5). The results of the present study differed from the earlier observation made by Liebert and Aster.⁹ They reported that serum HLA-B12 antigens were roughly proportional to HLA-B12 on platelets. The small number of subjects (N = 5) included in their study and the assay method used may account for the differences.

In conclusion, the results we report highly suggest that the chloroquine-elutable platelet HLA antigens may not be derived from plasma and that platelet HLA concentration is not in dynamic equilibrium with plasma HLA. Therefore,

the quantitative expression of specific HLA antigens and the immunogenicity of platelets most likely could not be altered by incubating platelets with selected plasma. Although plasma HLA may not be the source of adsorbed platelet HLA, our results do not exclude the possibility that most platelet HLAs indeed may be adsorptive in nature. Further studies on the surface orientation and the physicochemical properties of chloroquine-elutable HLA antigens are needed to identify the origin and the adsorptive nature of platelet HLA antigens.

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Non-Hodgkin's Lymphoma Containing Both B and T Cell Clones

By Eddie Hu, Lawrence M. Weiss, Roger Warnke, and Jeffrey Sklar

We describe a patient in whom two lymph node biopsies removed 18 months apart disclosed histologic and immunophenotypic evidence of a non-Hodgkin's lymphoma containing neoplastic lymphocytes of both B and T type. Analyses of immunoglobulin and T cell receptor genes confirmed the presence of separate B and T cell clones. In addition, immunogenotyping revealed the possibility of a second B cell clone within the patient's tumor. Development of a multiclonal lymphoma in this patient may relate

to the carcinogenic effects of chemotherapy or to a predisposition for neoplastic transformation of lymphocytes due to a previously diagnosed autoimmune condition. Another possible explanation is that the lymphoma implies the existence in this patient of a transformed lymphocyte-committed stem cell that is capable of generating both B and T lineage clones.

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MOST NEOPLASMS are believed to consist of a single clone of neoplastic cells. A possible exception to this rule has recently been described among non-Hodgkin's lymphomas. Within a subset of these neoplasms, two proliferating clones of neoplastic B lymphocytes have been found in individual patients.^{1,2} Distinction between the clones in these cases was based on differences detected in the configurations of DNA in rearranged immunoglobulin genes. The results of immunoglobulin DNA analysis did not preclude a common origin of both clones from a single progenitor cell in which the immunoglobulin genes had not yet undergone DNA rearrangement, however. Furthermore, the interpretation of biclonality among B cell lymphomas has been complicated by the discovery that rearranged immunoglobulin genes in some B cell tumors can undergo somatic mutation.³ Occasionally, these mutations may alter the position of bands in Southern blot autoradiograms, thereby simulating differences in gene rearrangements among individual cells from a monoclonal tumor.

The lymphomas so far presented as candidates for biclonality have included only neoplasms composed exclusively of B cells. We report here a case of non-Hodgkin's lymphoma in which separate clones of B and T lymphocytes were detected using a combination of immunogenotyping and immunophenotyping techniques. Because clones of both B and T lineage were present in this tumor, deductions about multiclonality in this case are not affected by somatic mutation of rearranged genes, a process limited to immunoglobulin genes of B cells. In addition, in different biopsy specimens from this case, two different B cell populations were identified in which no shared rearrangements of immunoglobulin genes could be found. Therefore, this tumor may contain at a minimum two, and possibly as many as three, apparently distinct clonal lines of lymphocytes, which may or may not be related through a common precursor.

MATERIALS AND METHODS

Case report. The patient is a 63-year-old white woman who presented in 1981 with symptoms of diffuse myalgias, especially in her arms and legs, associated with peripheral eosinophilia. Subsequently, she developed a diffuse erythematous rash with induration and thickening of the skin, predominantly on the trunk and extremities. A biopsy of the skin and soft tissues of the left thigh was performed, and the histologic diagnosis of eosinophilic fasciitis was made. The patient was treated with systemic steroids, resulting in reduction of symptoms and improvement in the cutaneous lesions.

In June 1983, the patient developed diffuse peripheral lymphadenopathy and was first seen at the Stanford University Medical

Center. Biopsy tissue from an enlarged left axillary lymph node was diagnosed as containing diffuse large noncleaved cell lymphoma (DLCL). Staging workup revealed a normal bone marrow biopsy, but the bipedal lymphangiogram demonstrated extensive involvement of retroperitoneal lymph nodes, and a chest x-ray film showed bilateral nodular lesions consistent with lymphoma. The patient was classified as a stage IVA DLCL and treated from June to November 1983 with six cycles of CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) chemotherapy, followed by the complete resolution of adenopathy and pulmonary nodules.

Soon after cessation of chemotherapy, the patient's initial skin lesions reappeared but responded to treatment with steroids combined briefly with small doses of cyclophosphamide. Late in 1984, she developed recurrent peripheral adenopathy. Biopsy of an enlarged left axillary lymph node in January 1985 revealed diffuse, mixed small and large cell lymphoma. Repeat staging indicated that her disease was confined to lymph nodes. Treatment was begun with BACOP (bleomycin, adriamycin, cyclophosphamide, oncovin, prednisone), producing a continued gradual regression of the patient's disease up to the present time.

Immunophenotype and immunogenotype studies. Biopsy tissues collected from the operating room were snap frozen in air-tight plastic capsules by immersion in a dry ice/isopentane bath and stored at -70°C . All tissues studied were obtained with the informed consent of the patient, in accord with institutional guidelines. Techniques for frozen-section immunoperoxidase studies have been described in detail elsewhere.⁴ Staining of frozen sections was performed using monoclonal antibodies directed against μ , κ , and λ immunoglobulin chains (Becton Dickinson, Mountain View, CA); Leu 1 through 5 (CD 2-5, 8) and 9 (CD 7) (Becton Dickinson); B1 (CD 20) (Coulter Clone, Hialeah, FL); 4G7 (CD 19) (Dr Ronald Levy, Stanford University); and TO15 (Dr David Mason, Oxford University).

DNA was extracted from tissue specimens and purified by standard procedures.^{5,6} Ten micrograms of DNA was digested with an appropriate restriction enzyme (New England BioLabs, Beverly,

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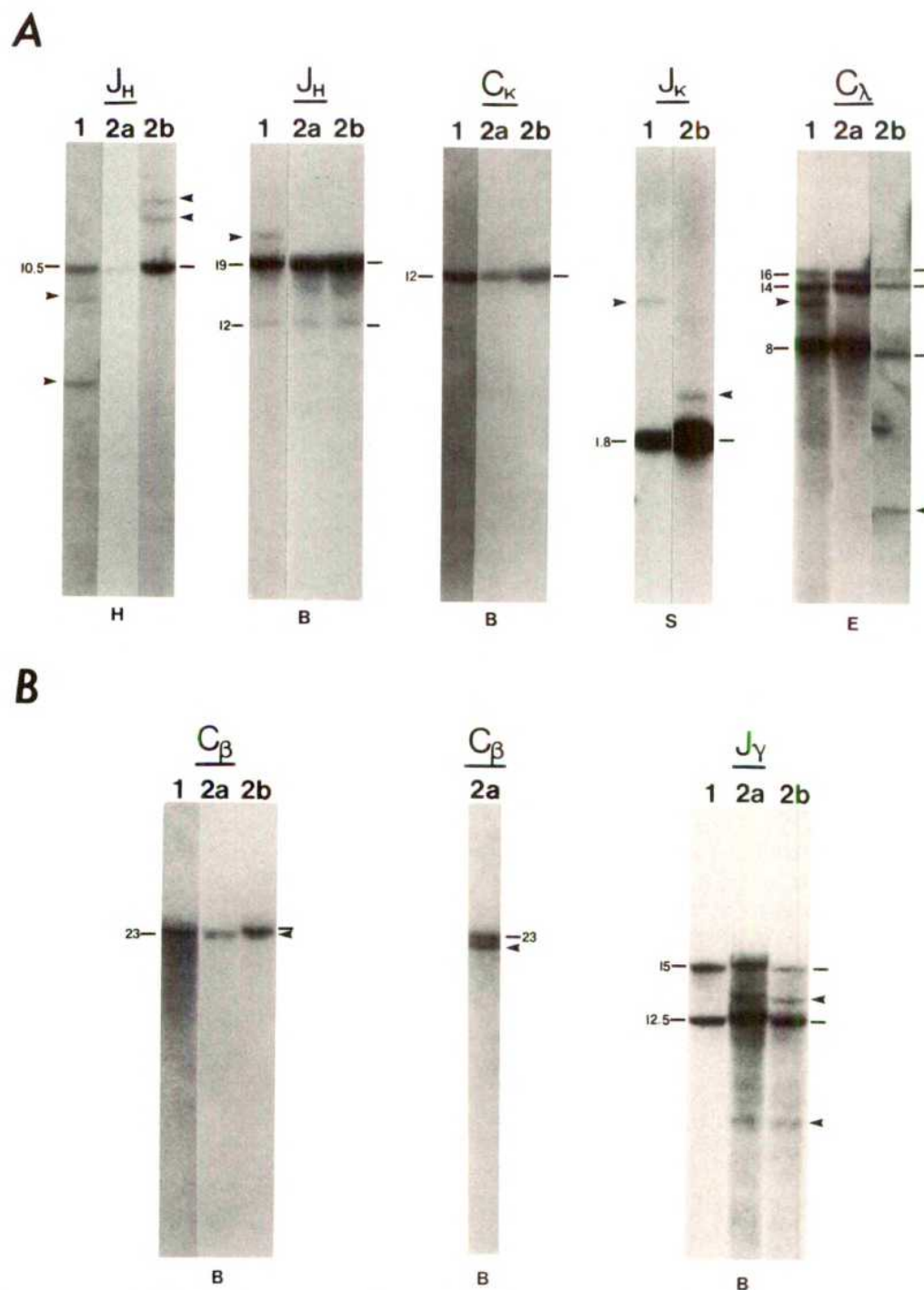


Fig 1. Analysis of immunoglobulin and T cell receptor gene DNA. Autoradiograms resulting from analyses for rearrangements of immunoglobulin heavy chain genes (A) and T cell receptor genes (B). The hybridization probes used are indicated above each panel. Lane 1 shows analyses of DNA from the initial (1983) biopsy specimen; lanes 2a and 2b show analyses of DNA from the two parts of the second (1985) biopsy specimen. Dashes indicate unarranged germline bands; size of the corresponding fragments is given in kilobases. Arrows indicate clonal, rearranged bands. The lanes in each panel were assembled from different analyses. Within a panel, the analyses were made under similar conditions (including length of electrophoresis) and were aligned using marker bands run in parallel for each analysis, but not shown. Letters below each panel represent the restriction enzymes used in the analyses: H, *HindIII*; B, *BamHI*; S, *SacI*; and E, *EcoRI*. The analysis in the middle panel of B is identical to that in the panel to the left, except that electrophoresis was allowed to proceed for about four times as long.

MA) and the resulting fragments were analyzed by the Southern blot hybridization procedure.⁷ DNA probe fragments were radiolabeled with α -[³²P]-dCTP by the random hexamer priming method.⁸ Genomic DNA fragments used for analysis were specific for the heavy chain joining region, the constant and joining regions of the κ light chain gene, the constant region of the λ immunoglobulin light chain gene, the constant region for the β T cell receptor gene, and the joining region of γ T cell receptor gene. The structures of each of these probes has been described in detail previously,^{2,6,9-12} as have been methods for hybridization and autoradiography.^{6,9}

RESULTS

Analysis of this patient's neoplasm was based on tissue obtained from two lymph node biopsies—the initial diagnostic biopsy performed in June 1983 and the biopsy performed at relapse in January 1985. Histologic review of the lymph node biopsy specimen from 1983 showed complete effacement of the normal lymph node architecture by a polymorphous population of atypical small and large lymphoid cells. Large cells with vesicular noncleaved nuclei predominated in most areas examined, leading to the classification of the tumor as an intermediate grade, diffuse large noncleaved cell lymphoma according to the International Working Formulation for classification of non-Hodgkin's lymphoma.¹³

Immunoperoxidase studies on fresh-frozen tissues from this lymph node demonstrated that the large atypical cells expressed the B lineage antigens TO15, B1 (CD 20), and 4G7 (CD 19); however, staining for immunoglobulin light chains or μ heavy chain was absent. Similarly, the T cell antigens Leu 1 through 5 (CD 2–5, 8) and 9 (CD 7) could not be detected on these cells. In contrast, many of the smaller lymphocytes stained for normal patterns of T cell antigens. The immunologic findings were interpreted as consistent with a B lineage lymphoma lacking immunoglobulin expression, surrounded by a polyclonal T cell host response.

The B cell lineage of the neoplasm in this biopsy specimen was confirmed by analysis of immunoglobulin gene rearrangements (Fig 1, lane 1). Two clonal rearranged bands were detected in the analysis of the heavy chain gene DNA with one enzyme (*Hind*III), and a single rearranged band with a second enzyme (*Bam*HI). One clonal rearrangement was found in an analysis of the λ light chain gene DNA. Analysis of DNA for the β and γ T cell receptor demonstrated bands only in the position of unrearranged germline DNA. The results obtained with this tissue specimen are consistent with a monoclonal B lineage lymphoma, in which both heavy chain alleles were probably rearranged and one of the two λ light chain alleles was rearranged.

The 1985 lymph node biopsy specimen showed different histologic features from those noted in the previous biopsy specimen. The tumor population again consisted of a polymorphous population of atypical small and large lymphoid cells, with a tendency for the larger cells to be found clustered around vessels (Fig 2). Because the number of small and large cells in this specimen appeared to be more even, the tumor was classified as a diffuse, mixed small and large cell lymphoma.

Immunoperoxidase studies on the 1985 lymph node suggested two distinct subpopulations (Fig 3). Cells in some

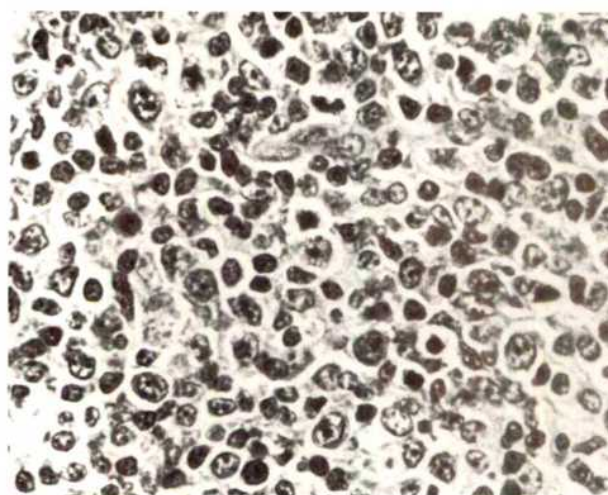


Fig 2. Histologic appearance of the second (1985) lymph node biopsy specimen. The lymphoma varied in its morphology from field to field but generally consisted of a diffuse proliferation of atypical small and large lymphocytes. The specimen from this biopsy was diagnosed as a diffuse, mixed small cleaved and large cell lymphoma. (Hematoxylin and eosin stain, original magnification $\times 640$; current magnification $\times 475$.)

areas within the sections from this node, particularly in those areas adjacent to vessels, showed expression of the B lineage antigens TO15, B1 (CD 20), and 4G7 (CD 19), but did not express T cell antigens. This cell population failed to bind anti- λ , κ , or μ immunoglobulin antibodies. Cells in other areas of the sections expressed the T cell antigens Leu 1, 3, 4, and 5 (CD 2–5). Only scattered cells expressed Leu 2 (CD 8). Staining for Leu 9 (CD 7) on these cells was variable, from absent to moderate intensity on different cells. The immunologic findings in this lymph node biopsy specimen were interpreted as suggesting both a B cell lymphoma, based on the presence of immunoglobulin-negative B lineage cells, and a T cell lymphoma, based on the monotypic helper phenotype with abnormal, inconsistent staining for Leu 9 (CD 7).

Lymph node tissue from the 1985 biopsy had been divided into two parts (a and b) immediately after removal from the patient; tissue fragments representing the two parts of this specimen were separately analyzed for gene rearrangements (Fig 1, lanes 2a and b). Separate immunophenotypic studies were not available on these two portions of the 1985 biopsy. No clonal rearranged bands were detected in analyses of any immunoglobulin genes prepared with DNA extracted from tissue of part a. Analysis of the β T cell receptor gene demonstrated two closely migrating bands, however, one in the germline position and a second band slightly below the germline band. Analysis of γ T cell receptor genes in this tissue showed two rearranged bands, in addition to unrearranged, germline bands. Therefore, these data indicate a clonal population of T cells within this part of the specimen.

Examination of DNA from tissue of part b of the 1985 biopsy specimen produced different results. This tissue showed two clonal rearrangements in analyses of the immunoglobulin heavy chain gene with one enzyme (*Hind*III), and no detectable rearrangements with the other (*Bam*HI). A

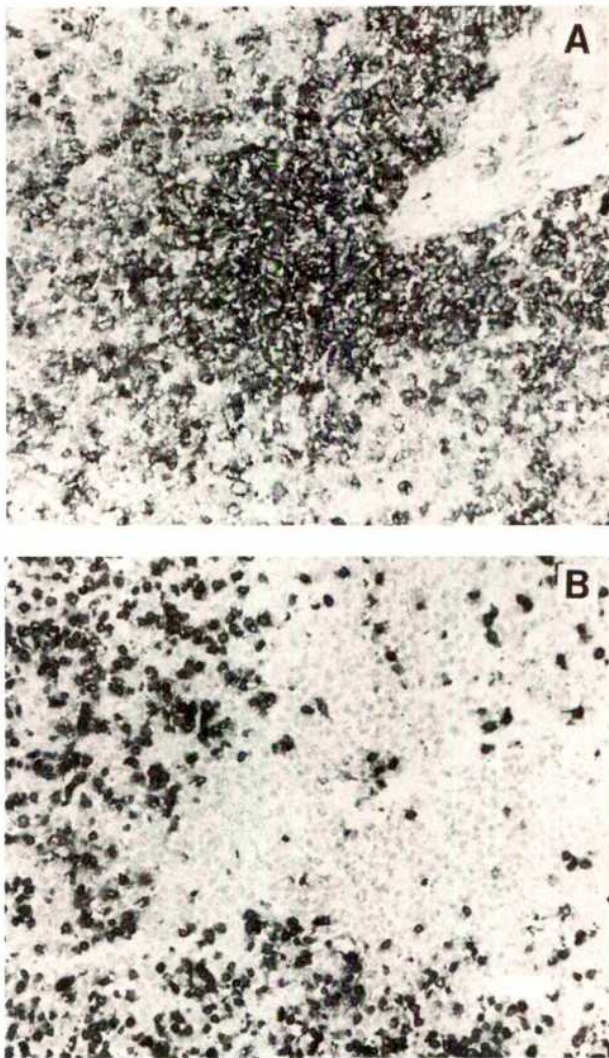


Fig 3. Immunophenotype of the second (1985) lymph node biopsy specimen. In most areas of the specimen, the larger cells tended to cluster around vessels. Staining of the larger cells for the B lineage antigen 4G7 (CD 19) is apparent (A).¹⁴ Cells bearing the 4G7 antigen are surrounded by a dark ring of peroxidase reaction product. Other stains showed these cells to express other B lineage antigens, but not κ , λ , or μ immunoglobulin. Staining in a section similar to that in A of many of the smaller lymphocytes for the pan-T cell marker Leu 4 (CD 3) is apparent (B). These cells also expressed the pan-T cell antigens Leu 1, 3 and 5 (CD 2, 4, and 5), and showed anomalous staining for the T cell marker Leu 9 (CD 7) and occasional staining for Leu 2 (CD 8). (Original magnification of both A and B $\times 160$; current magnification $\times 145$.)

single clonal rearrangement was found for the λ light chain gene. Analyses of the β and γ T cell receptor genes produced results similar to those seen for part a. These findings imply that the T cell clone in part b was admixed with a B cell clone in part b.

None of the rearranged immunoglobulin gene bands detected in part b of the 1985 tissue matched those found in the earlier biopsy specimen, suggesting the emergence of a second clonal population of B cells in the patient. As a further test of the non-identity of the B cell populations, we investigated the configurations of DNA in the κ immuno-

globulin locus of these two tissues using a hybridization probe for the κ gene joining region.¹⁰ Although the κ constant regions in the clonal population of both specimens had apparently been deleted, the κ joining regions were retained and produced bands in different positions between the two tissues, as detected in analyses with *Sac*I, *Bam*HI, and *Hind*III enzymes (the latter two analyses not shown).

DISCUSSION

The histology and immunophenotyping of the two lymph node tissue samples in this case support the diagnosis of a non-Hodgkin's lymphoma containing B and T cell clones. DNA analyses confirmed the presence of separate clonal B and T cell populations in the second biopsy specimen. In addition, a possible distinction between the B cell clone in the first biopsy sample and the B cell clone in the later biopsy sample was demonstrated by analysis of DNA.

Past reports have described coexistent rearrangements of T cell receptor genes and immunoglobulin genes in the same cells within some lymphoid neoplasms.¹⁵⁻¹⁹ This cannot be the situation in the present case. Clonal rearrangements of the T cell receptor genes and immunoglobulin genes were both found in part b of the 1985 biopsy. Part a, however, contained only T cell receptor gene rearrangements in the absence of immunoglobulin gene rearrangements. The conclusion that T cell receptor and immunoglobulin gene rearrangements occurred in different cells is consistent with the immunophenotypic data, which showed separate B and T lineage populations among the histologically atypical cells of tissue sections.

On first appraisal, failure to detect any common immunoglobulin gene rearrangements between the two clonal B cell populations in this patient suggests that the two populations are unrelated or at least arose from a common precursor which lacked rearranged genes. As described previously, however, postrearrangement somatic mutations in immunoglobulin DNA may change the position of bands derived from a single recombination of immunoglobulin gene segments. Such changes may be extensive, affecting several different genes within a single monoclonal tumor (M.L. Cleary and J. Sklar, unpublished observations). In this regard, the immunophenotyping and DNA studies demonstrated noteworthy similarities among the two B cell populations in this patient. Both clones failed to express detectable levels of cellular immunoglobulins, a feature associated with $\sim 15\%$ of all large cell lymphomas. In addition, both populations showed rearrangements of lambda light chain genes, an occurrence in less than one-third of monoclonal B cell tumors.

On the other hand, extensive somatic mutation of immunoglobulin genes has so far been documented only in follicular B cell lymphomas, not in large cell lymphomas, such as in this patient. Moreover, differences in detectable rearrangements in this case extend even as far as the nonfunctional κ -genes that have undergone partial deletion. At present, it is not known if such nonfunctional genes are subject to somatic mutation, but whether or not these two apparently different B cell clones are actually mutational variants of the same

clone does not detract from the conclusion that a separate T cell clone was present within this patient's tumor.

Although the results of DNA analyses offer strong evidence for at least two different clones of lymphocytes in this patient's neoplasm, they do not specify the neoplastic nature of these clones. Could only one of the clones actually be neoplastic while the others represent immunologic reactions against the neoplastic clone? This seems unlikely for at least two reasons. First, detectable clonal proliferations of lymphocytes seem to be unusual outside of malignancy^{6,11} and have not previously been associated with immunological responses. Second, the immunophenotype of each clone was abnormal: Both B cell clones lacked immunoglobulin expression, and the T cell clone showed an anomalous, monotypic pattern of T cell antigens commonly found in peripheral T cell lymphomas.²⁰ In addition, the absence of detectable surface or cytoplasmic immunoglobulin from the B cell clones argues against their role in a normal immune response.

Several mechanisms could explain the occurrence of multiple clones of lymphocytes within this patient's tumor. One obvious possibility is that it is the result of a very rare coincidence in which two or three lymphocytes were independently transformed to malignancy more or less simultaneously. An interesting feature of this case, however, is that the patient initially had the rare autoimmune skin disorder, eosinophilic fasciitis.²¹ Lymphomas appear with greater than average frequency among patients with certain other autoimmune disorders, such as celiac disease,²² Sjögren's disease,²³ and chronic lymphocytic thyroiditis.²⁴ In addition, one patient with diffuse fasciitis has been described in whom a lymphoproliferative disorder was diagnosed at autopsy.²⁵ Therefore, one or more of the neoplastic lymphocytic clones detected within our patient may have developed as a result of a high rate of lymphocytic transformation due to autoimmunity.

Another possible cause for multiple clones in this patient's lymphoma is the chemotherapy she received after the diagnosis of her first neoplastic B cell clone. Most second neoplasms arising after chemotherapy are acute nonlymphocytic leukemias, although non-Hodgkin's lymphomas are also overrepresented among second tumors.²⁶⁻²⁸ The greatest increase in relative risk appears to occur among patients treated by combined radiation and chemotherapy. The interval from therapy to detection of the second cancer averages

between 3 and 10 years. Given these facts, the chance that our patient's therapy for her initial clone could have induced either or both of the other two clones does not seem great since she received neither radiation nor particularly intensive chemotherapy at any time. Moreover, the interval of ~18 months from the beginning of chemotherapy to diagnosis of the second and third clones was shorter than that in most cases of posttherapy second tumors.

Any speculation about chemotherapy-induced tumors in this patient must take into consideration that there are no published studies of second neoplasms in non-Hodgkin's lymphoma patients who have received the same CHOP regimen administered to the patient in this study. Furthermore, the ability to distinguish different clones of cells among neoplastic lymphocytes is a relatively recent development. Consequently, in the past, additional clones of neoplastic lymphocytes induced by therapy for non-Hodgkin's lymphoma would probably have been regarded as a relapse attributable to reemergence of the original clone. Therefore, induction of additional clones of neoplastic lymphocytes by chemotherapy may be more common in non-Hodgkin's lymphoma than is currently recognized.

A final possible mechanism to account for this patient's lymphoma is derivation from a single transformed early lymphocytic precursor. Lymphocyte-committed stem cells capable of generating both B and T cells are presumed to exist, although the evidence accumulated to date for this lymphocyte precursor is indirect. Among the best indications for the existence of such a cell is the deficiency of both B and T cells in patients with severe combined immune deficiency. More recently, the discovery of certain lymphocytic tumors containing a clone of cells that possess both immunoglobulin and T cell receptor gene rearrangements further supports existence of a lymphocytic stem cell. The possibility that the B and T cell clones in the present case of lymphoma arose from a single transformed cell may provide additional indirect evidence for a stem cell with pluripotential capacity for differentiation along both B and T cell lines.

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Equal Stabilities of Normal β Globin and Nontranslatable β^0 -39 Thalassemic Transcripts in Cell-Free Extracts

By C.A. Stolle, M.S. Payne, and E.J. Benz, Jr

Patients with β^0 thalassemia arising from premature terminator codon mutations in the gene for β globin do not produce β globin protein; these individuals also exhibit a decreased amount of β globin mRNA in their erythroid cells. The absence of β globin protein is readily explained by the inability of the β^0 -39 mRNA to be translated. The decrease in β globin mRNA has been attributed to either decreased cytoplasmic stability of the nontranslatable mRNA or to an undefined nuclear lesion. To compare directly the relative stabilities of normal and β^0 -39 thalassemic globin transcripts, we prepared normal and thal-

semic β globin pre-mRNAs and mRNAs using cloned DNA templates and the SP6 promoter-polymerase system. The stability of the transcripts was assessed by incubation in various cell-free extracts. Our results indicate that although the stabilities of the β globin transcripts varied considerably from one extract to another the stabilities of the β^0 -39 thalassemic pre-mRNAs and mRNAs were equal to those of normal β globin mRNAs in every extract tested.

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THE β THALASSEMIAS are inherited disorders characterized by reduced (β^+ thalassemia) or absent (β^0 thalassemia) synthesis of the β globin subunit of normal adult hemoglobin. Molecular cloning and nucleotide sequencing of numerous β -thalassemia genes have revealed a variety of mutations that impair β globin gene function in patients with β thalassemia. These mutations include partial gene deletions, impaired function of the transcription promoter region, errors in processing of β mRNA precursors (due either to alteration of the normal splice sites or to activation of "cryptic" splicing sites), and creation of premature translation termination codons that abolish translation of β^0 mRNA into β globin.^{1,2}

A translation termination mutation occurring at codon 39 (CAG $\rightarrow$ UAG) is a common cause of β^0 thalassemia among Greek and Italian patients.³⁻⁷ The β^0 thalassemia phenotype in homozygotes is clearly due to the inability of the β^0 -39 mRNA to be translated into β globin; however, the repeated observation^{4,6,8,9} that there is also a marked quantitative reduction in the amount of β globin mRNA in these patients' erythroid cells is not readily explained solely by the lesion in translation arising from this mutation. One frequently invoked hypothesis is that the inability of these mRNAs to be translated leads to accelerated nucleolytic degradation (instability) in the cytoplasm.^{4,10} Other possibilities include the presence of undetected mutations in sequences flanking the gene that affect transcription or processing, impaired nuclear to cytoplasmic transport, or decreased stability of the mRNA in the nucleus.^{11,12}

To address the question of whether normal and β^0 -39-thalassemic transcripts differ in their stability in the cytoplasm, we incubated synthetically prepared normal and thalassemic transcripts in various cell-free extracts. In this report, we describe the relative stabilities of these transcripts in vitro and discuss the lesion responsible for the decreased level of β globin mRNA in β^0 -39 thalassemia.

METHODS

Source of cells and cell-free extracts. Mouse erythroleukemia (MEL) cells (strain 745A) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) (GIBCO, Grand Island, NY), 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 2 mmol/L of L-glutamine (GIBCO), at 37°C in a 5% CO₂ atmosphere. Cultures were split every 3 to 5 days to maintain a cell density of 1×10^4 to 1×10^6 cells/mL. HeLa cells

were grown to a density of 5×10^5 cells/mL in suspension culture in Spinner medium (KC Biologicals, Lenexa, KS) supplemented with 10% FCS, penicillin and streptomycin, and L-glutamine. Suspension cultures were flushed with 10% CO₂/90% air, capped, and incubated at 37°C.

Rabbit reticulocyte lysate translation reaction mixture (Bethesda Research Laboratories, Gaithersburg, MD) was prepared according to manufacturer's instructions. A HeLa whole cell extract capable of in vitro synthesis and splicing of pre-mRNAs was purchased from Bethesda Research Laboratories (eucaryotic In Vitro transcription system) and used according to manufacturer's directions, or prepared from HeLa cells and used according to the methods of Manley and colleagues.¹³ Nuclear and cytoplasmic extracts of MEL cells were prepared according to the method described by Dignam and co-workers¹⁴ for preparation of in vitro transcription lysates. Alternatively, cytoplasmic extracts of MEL cells were prepared by nitrogen cavitation¹⁵ to minimize release of lysosomal ribonucleases.

Plasmid construction. Construction of SP6 plasmids containing human β globin genes or cDNA sequences was accomplished as outlined in Fig 1. Plasmid pSPk β c, (a gift of Dr R. Spritz), was used as the parent vector. This plasmid contains a full-length, normal β globin cDNA plus SV40 sequences coding for a 175-base pair (bp) poly A tail. Transcripts prepared from pSPk β c are identical to naturally occurring β globin mRNA except for the presence of 20 non-globin bases in the 5' flanking region and a small number of bases in the 3' flanking region between the poly A tail and the HindIII restriction site.

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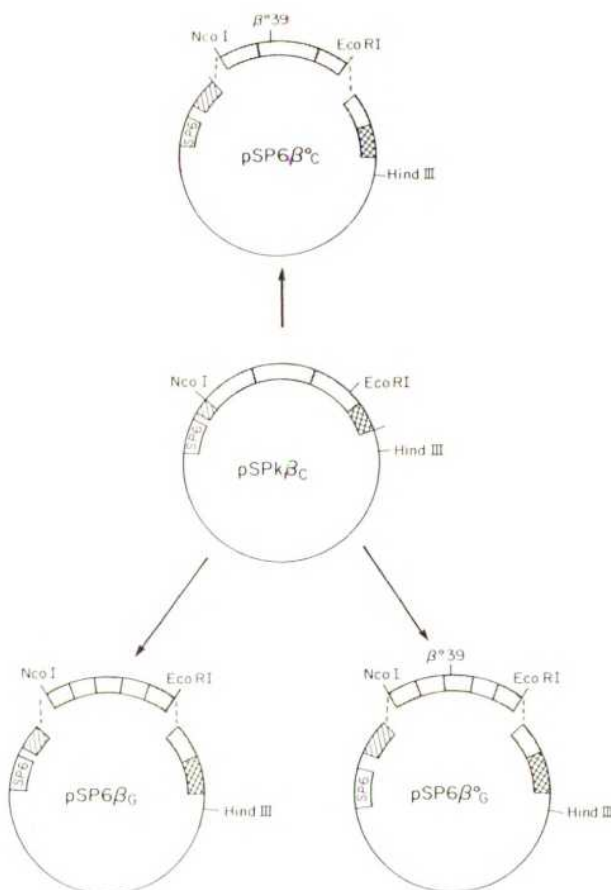


Fig 1. Construction of plasmids. Plasmid pSPk β C contains the binding site for SP6 RNA polymerase (SP6), the coding region of a full-length normal β globin gene (shaded boxes), 20 bp of 5' flanking region as a result of cloning strategy (striped boxes), 175-bp sequence of SV40 DNA specifying a poly A tail (cross-hatched boxes), and a unique HindIII site. SP6 plasmids containing a β° -39 thalassemic cDNA or normal and β° -39 thalassemic genomic sequences were prepared by ligating the appropriate NcoI/EcoRI fragments into pSPk β C digested with NcoI and EcoRI. Intron sequences in the genomic clones are represented by open boxes.

Plasmids containing normal β globin or β° -39 thalassemic gene sequences were prepared by ligating a 1.35-kilobase (kb) NcoI/EcoRI fragment from appropriate subclones of normal β globin genes¹¹ into pSPk β C digested with NcoI and EcoRI. Likewise, an SP6 plasmid containing a β° -39 thalassemic cDNA was obtained by subcloning a 371-bp NcoI/EcoRI fragment from a β° -39 cDNA clone¹⁶ into pSPk β C digested with NcoI and EcoRI. The presence in the β° -39 parent plasmids of a C $\rightarrow$ T mutation at the codon for amino acid 39 was previously verified by DNA sequence analysis.^{11,16} All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA).

Synthesis and Capping of SP6 transcripts. Labeled RNA transcripts were prepared according to the method of Green and colleagues.¹⁷ In brief, plasmid DNA was linearized with HindIII and transcribed at 37°C for 1 hour with SP6 RNA polymerase (New England Nuclear, Boston) in 40 mmol/L of Tris buffer (pH 7.5) containing 6 mmol/L of MgCl₂, 2 mmol/L of spermidine, 10 mmol/L of NaCl, 10 mmol/L of (DTT), RNasin (1 U/ μ L; Promega Biotech, Madison, WI), 500 μ mol/L of each rNTP, and 100 μ Ci of [³²P]-GTP (400 mCi/mmol; Amersham, Arlington Heights, IL). The DNA template was removed by digestion with RNAase-free DNAase (25 μ g/mL) for 15 minutes at 37°C. The RNA was then extracted with phenol:chloroform, centrifuged through a Sephadex G-50 column to remove unincorporated label, ethanol-precipitated, and resuspended in sterile distilled water. Typical yields were RNA 2 to 6 μ g/ μ g of DNA, with a specific activity of 1.7×10^7 dpm/ μ g RNA.

A 5'-terminal "CAP" structure [m⁷G(5')ppp(5')N] was added to the SP6 RNA transcripts with guanylyltransferase (1 U/ μ g RNA; Bethesda Research Laboratories) in a 30- μ L reaction containing 50 mmol/L of Tris-HCl (pH 7.9), 1.25 mmol/L of MgCl₂, 6 mmol/L of KCl, 3 mmol/L of DTT, RNasin (1 U/ μ L), 100 μ mol/L of S-adenosyl methionine, and 300 μ mol/L of GTP. After incubation at 37°C for 60 minutes, the reaction was extracted with phenol:chloroform and precipitated with ethanol. The resuspended transcripts migrated as a single band of either 1.8 kb (pre-mRNA) or 0.7 kb (mRNA) on denaturing agarose gels. The efficiency of the CAPPING reaction was estimated by determining survival of full-length RNA transcripts in a HeLa whole cell extract (Fig 2). Under the conditions used for these measurements, non-CAPPED mRNA is rapidly degraded but CAPPED mRNA is completely preserved. For the experiments described in this report, only preparations exhibiting 80% to 100% stability in HeLa extract after CAPPING were used. Other methods of capping, such as inclusion of CAP analogues in the polymerization reaction, did not result in efficient synthesis of

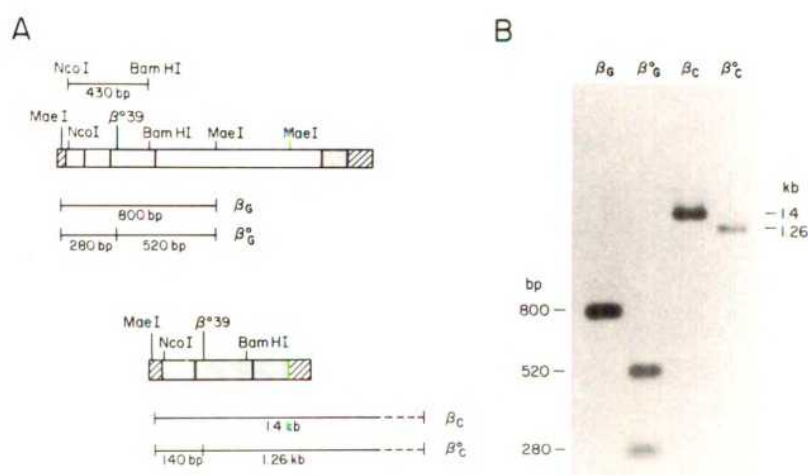


Fig 2. Stability of RNA transcripts $\pm$ 5' terminal CAP in HeLa cell extract. Equal amounts of CAPPED and non-CAPPED RNA transcripts were incubated at 30°C in a HeLa whole cell extract prepared according to the method of Manley and colleagues.¹³ Samples removed at the time points indicated were processed for electrophoresis as described in Fig 4. Panel A, pre-mRNA; panel B, mRNA; β cap and β° cap, CAPPED normal and thalassemic transcripts; β and β° , non-CAPPED normal and thalassemic transcripts; M, RNA size markers. Aliquots from incubations containing pre-mRNAs (panel C) or m-RNAs (panel D) were taken at the time points indicated and spotted onto DE-81 filters. The filters were washed successively with 0.5 mol/L of Na₂HPO₄, dH₂O, and ethanol, and then dried and counted in a scintillation counter. The counts were plotted as a percentage of the input RNA remaining v time.

full-length transcripts. RNAs modified at the 5' end are referred to in this article as CAPped; RNAs not modified in this way are referred to as non-CAPped. The RNA transcripts were synthesized with poly A tails and 5' CAPS since both are naturally occurring modifications believed to affect mRNA stability.

Stability in cell-free extracts. The stability of the labeled transcripts was assessed by incubating equal amounts of RNA in cell-free extracts for up to 2 hours. A 2-hour incubation period was chosen on the basis of earlier studies from our laboratory,¹¹ that showed that intact erythroid cells from a patient with a β^0 -39 thalassemia gene exhibited a reduction of newly synthesized β globin mRNA to steady-state thalassemic levels within 20 minutes after exposure to [³H]-uridine. Therefore, any mRNA turnover relevant to whole cells should occur well within our 2-hour observation period.

A typical assay for in vitro stability consisted of 3×10^5 cpm of RNA (20 ng) in 30 μ L of cell extract. Aliquots taken at time points during the incubation were diluted in 300 μ L of TE buffer (10 mmol/L of Tris-HCl, pH 7.5; 1 mmol/L of EDTA), extracted with phenol:chloroform, ethanol precipitated, and resuspended in a small volume of sterile dH₂O. The samples were mixed with 3 vol of a denaturing buffer containing 50% deionized formamide, 17% formaldehyde, and 1x MOPS buffer (40 mmol/L of morpholinopropanesulfonic acid, pH 7.0; 10 mmol/L of sodium acetate; 1 mmol/L of EDTA), heated to 65°C for 10 minutes, and electrophoresed on a 1.5% agarose gel containing 6.7% formaldehyde in 1x MOPS buffer. The labeled RNA bands were transferred to nitrocellulose paper by a Northern blotting procedure,¹⁸ and the blots were exposed to x-ray film (Kodak XAR-5). To quantitate the stability of labeled RNA in the cell extracts, aliquots were spotted directly onto Whatman DE-81 filters, washed six times with 0.5 mol/L of Na₂ HPO₄, twice with dH₂O, and twice with 95% ethanol. The filters were then air dried and counted in a scintillation counter. The stability of the RNA was plotted as the percentage of labeled RNA (cpm) bound to the filter v time.

Cell-free translation. CAPped normal β globin and β^0 -39 thalassemic mRNAs (200 to 500 ng) were translated in a rabbit reticulocyte lysate in vitro translation system (Bethesda Research Laboratories) according to the manufacturer's specifications. The translation products were labeled with either [³⁵S]-methionine (5 μ Ci/reaction) or [³H]-leucine (5 μ Ci/reaction). The labeled proteins were electrophoresed on a 12.5% polyacrylamide slab gel containing 6 mol/L of urea, 2% Triton X-100, and 5% acetic acid^{19,20} along with unlabeled human globin chains as markers. The gel was either stained with Coomassie blue and destained in 5% acetic acid and 25% methanol or processed for fluorography with Enlightening (New England Nuclear), dried on a slab gel dryer, and exposed to x-ray film.

Splicing of normal β globin and β^0 -39 thalassemic pre-mRNAs. CAPped normal β globin and β^0 -39 thalassemic pre-mRNAs were incubated for 4 hours at 30°C essentially as described by Krainer and associates²¹ in an in vitro splicing extract prepared from HeLa cells by the method of Dignam and associates.¹⁴ The splicing products were electrophoresed on a denaturing 5% polyacrylamide gel and visualized by autoradiography.

RESULTS

Verification of SP6 plasmid construction. Construction of SP6 plasmids containing normal β globin and β^0 -39 thalassemic globin sequences was verified by digestion with the restriction enzyme Mae I. Mae I recognizes the sequence C/TAG; a new Mae I site is created by a single C $\rightarrow$ T conversion at the codon for amino acid 39 in exon 2 of the β globin gene. The position of relevant Mae I sites and the size

of the expected digestion fragments is diagrammed in Fig 3A. All constructs (normal and β^0 thalassemic pre-mRNAs and mRNAs) were digested with Mae I, and the products were electrophoresed on a 1% agarose gel. Southern blot analysis of the digestion products using a nick-translated 430-bp NcoI/BamHI probe specific for sequences spanning the β^0 -39 mutation is shown in Fig 3B. As expected, digestion of the normal β globin gene construct produced an 800-bp band; the β^0 -39 thalassemic gene construct yielded bands at 520 bp and 280 bp instead of the 800-bp fragment, reflecting the presence of the new Mae I site introduced by the β^0 -39 mutation. Similarly, digestion of β globin cDNA constructs resulted in a 1.4-kb band from pSPk β c or a 1.26-kb band from pSP6 β^0 c.

Stability of RNA transcripts in a rabbit reticulocyte lysate. Equal amounts of CAPped and non-CAPped, normal β , and β^0 -39 thalassemic transcripts were incubated in a translationally competent rabbit reticulocyte lysate. The β globin transcripts were largely stable in the reticulocyte lysate, since considerable amounts of full-length transcripts were present even after 2 hours of incubation (Fig 4). Some RNA degradation did occur, however, resulting in the loss of 15% to 25% of the labeled RNA as measured by DE-81 filter counts (data not shown). There was no difference in stability

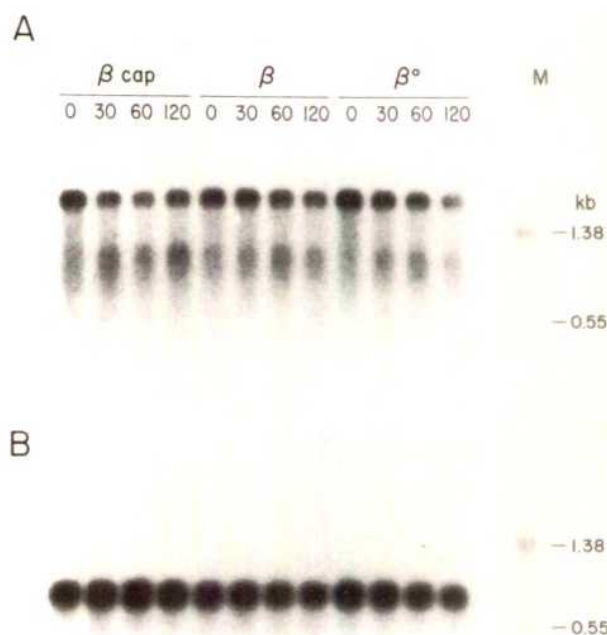


Fig 3. Digestion of β globin constructs with Mae I. Construction of the SP6 plasmids containing normal β and β^0 -39 thalassemic globin sequences was verified by digestion with Mae I. Schematic diagram showing position of Mae I sites in the β globin gene and cDNA plasmid inserts, the size of the fragments expected on digestion of the normal β and β^0 -39 plasmid constructs with Mae I, and the origin of a 430-bp NcoI/BamHI probe (A). Southern blot of Mae I digest hybridized to nick-translated 430-bp NcoI/BamHI fragment from the β globin gene (B). β_0 and β^0 , fragments derived from the normal β globin or β^0 -39 thalassemic gene sequences, respectively; β_0 and β^0 , fragments derived from the normal or thalassemic cDNA sequences, respectively.

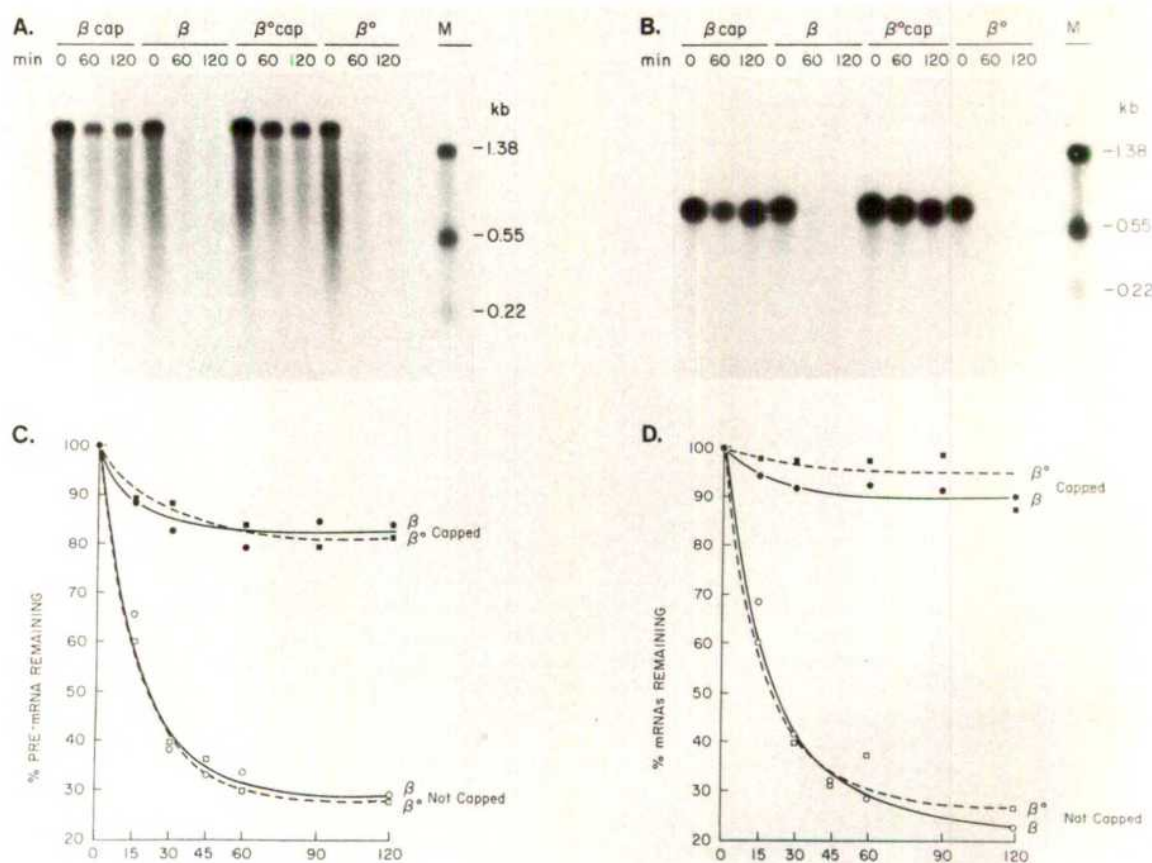


Fig 4. Stability of RNA transcripts in rabbit reticulocyte lysate. Equal amounts of normal and β° -39 thalassemic transcripts were incubated in a rabbit reticulocyte lysate translation mixture for 2 hours at 30°C. Aliquots were removed after 0, 30, 60, and 120 minutes, extracted with phenol:chloroform, precipitated with ethanol, and resuspended in a small volume of sterile dH_2O . The samples were denatured and electrophoresed on a 1.5% agarose gel containing formaldehyde. The mRNA was transferred to nitrocellulose paper, and the filter was exposed to x-ray film. The faint band of labeled material migrating below full-length pre-mRNA is an artifact of electrophoresis seen occasionally in reticulocyte extracts. Panel A, pre-mRNA; panel B, mRNA; β cap, CAPped normal β globin transcript; β , non-CAPped normal β globin transcript; β° , non-CAPped β° -39 thalassemic transcript; M, RNA size markers.

between normal β globin and β° -39 thalassemic pre-mRNAs (Fig 4A) or mRNAs (Fig 4B).

The stability of the RNA transcripts in reticulocyte extract was independent of translational activity by several lines of evidence. In addition to the β° -39 transcripts, other nontranslatable messages, such as pre-mRNAs (containing introns) and non-CAPped mRNAs (unable to interact with translation initiation factors), were also as stable in the rabbit reticulocyte translation mixture as were translatable CAPped mRNAs (Fig 4). Furthermore, all RNA transcripts were equally stable in a rabbit reticulocyte lysate prepared without creatine phosphate and the amino acids required for translation (data not shown).

Stability of RNA transcripts in a HeLa whole cell extract. In a HeLa whole cell extract, prepared as an *in vitro* transcription extract by the method of Manley and co-workers,¹³ a difference in stability between CAPped and non-CAPped transcripts was evident. CAPped pre-mRNAs (Fig 2A) and mRNAs (Fig 2B) were relatively stable for up to 2 hours in the HeLa cell extract whereas non-CAPped transcripts were readily degraded. Autoradiographs of dena-

turing agarose gels revealed that no full length non-CAPped transcripts remained after 30 minutes of incubation. The stability of CAPped transcripts in the HeLa cell extract was not dependent on protection from ribonuclease attack by polysome formation (ie, translation), since the extract is prepared by ammonium sulfate precipitation of nuclear and cytoplasmic proteins and is devoid of ribosomes. More important, there was no difference in the stability of CAPped normal or thalassemic transcripts or in the rate of degradation of non-CAPped normal or thalassemic transcripts as assessed by DE-81 filter counts (Fig 2C and D). Although the HeLa cell extract clearly had the potential to degrade RNA transcripts, no difference in stability was detected between normal β globin and β° -39 thalassemic transcripts.

Stability of RNA transcripts in MEL cell extracts. Nuclear and cytoplasmic extracts of MEL cells were prepared by several methods to examine the stability of globin transcripts in extracts reflecting an erythroid progenitor cell environment. The stability of β globin transcripts in the MEL cell extracts varied with the method of preparation. For example, the stability of β globin transcripts in nuclear

extracts of MEL cells prepared by the method of Dignam and associates¹⁴ or cytoplasmic extracts of MEL cells prepared by nitrogen cavitation was comparable to that of β globin transcripts in rabbit reticulocyte lysates (data not shown).

In contrast, each of the RNA transcripts exhibited rapid degradation (as assessed by DE-81 filter counts) in an S-100 cytoplasmic extract¹⁴ prepared from uninduced MEL cells. Both pre-mRNAs (Fig 5A) and mRNAs (Fig 5B), regardless of 5' CAP status, were degraded. There was no difference in stability between normal β globin and β^0 -39 thalassemic transcripts, even at time points <20 minutes. Although 50% of the input RNA was still able to bind to DE-81 filters after 60 minutes of incubation, no full-length message remained (Fig 6, MELC). This result contrasts with the relative preservation of full-length RNA observed after a comparable exposure to reticulocyte lysate (Fig 6, RRL).

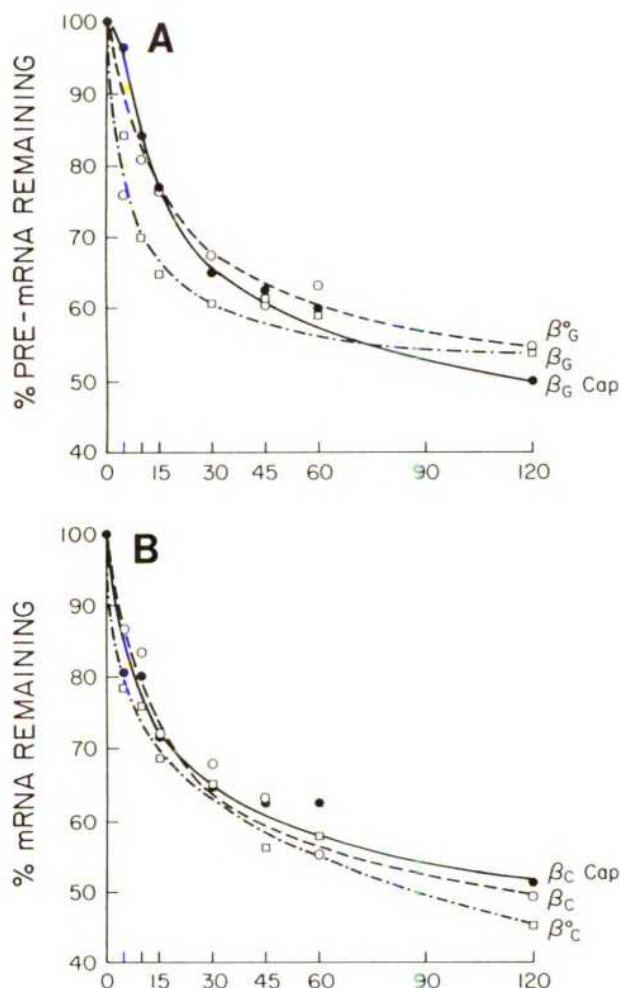


Fig 5. Stability of pre-mRNAs and mRNAs $\pm$ 5' terminal CAP in MEL cell extract. An S-100 cytoplasmic fraction was prepared from MEL cells by the method of Dignam and colleagues.¹⁴ The stability of labeled RNAs was assessed by DE-81 filter counts as described in Fig 4. Pre-mRNAs (A); mRNAs (B); β_G CAP and β_G CAP, CAPped normal β globin transcripts; β_G and β_G , non-CAPped normal β globin transcripts; β_G^0 and β_G^0 , non-CAPped thalassemic transcripts.

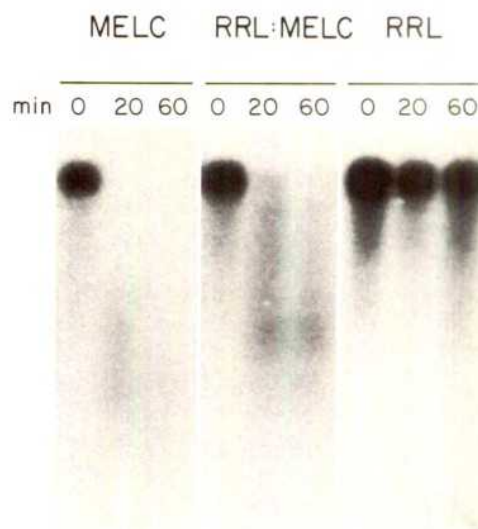


Fig 6. Stability of SP6 transcripts in MEL cell cytoplasmic extracts after preincubation in rabbit reticulocyte lysate translation mixture. CAPped β globin mRNA was preincubated in a rabbit reticulocyte lysate for 15 minutes before the addition of an equal volume of MEL cell extract. Aliquots taken at time points after addition of the cytoplasmic extract were electrophoresed on a denaturing agarose gel as described in Fig 3. MELC, mRNA incubated in MEL cell extract alone; RRL:MELC, mRNA preincubated in a rabbit reticulocyte lysate before addition of MEL cell extract; RRL, mRNA incubated in rabbit reticulocyte lysate alone.

S-100 cytoplasmic extracts prepared from MEL cells induced with dimethylsulfoxide (DMSO) or hemin degraded RNA transcripts as rapidly as did cytoplasmic extract from uninduced MEL cells (data not shown). Addition of up to 16 U/ μ L of RNasin failed to inhibit the ribonuclease activity of the MEL cell extract. In no case did we ever encounter any differences in stability between normal β globin and β^0 thalassemic transcripts in any of the MEL cell extracts tested.

Because the MEL cell S-100 extract lacked ribosomes, we examined whether prebinding the mRNAs to ribosomes would improve mRNA stability in this extract. Preincubating CAPped mRNAs in a fully active rabbit reticulocyte translation mixture before adding MEL cell cytoplasmic extract failed to enhance the survival of the mRNAs substantially (Fig 6). The stability (or lability) of the mRNAs was, therefore, independent of translational activity in this extract.

Translation of normal β and β^0 -39 thalassemic mRNAs. To demonstrate the activity of the rabbit reticulocyte lysate and the translatability of the synthetic β globin transcripts, we incubated CAPped normal β globin and β^0 -39 thalassemic mRNAs in a rabbit reticulocyte lysate in vitro translation system and labeled the newly synthesized peptides with either [³H]-leucine or [³⁵S]-methionine. The translation products were mixed with unlabeled human globin chains and separated by electrophoresis in a 12.5% polyacrylamide gel containing Triton X-100 (New England Nuclear), acetic acid, and urea.^{19,20} This electrophoretic system separates proteins by charge and not necessarily by molecular size.

Normal β globin mRNA promoted synthesis of a protein that comigrated with normal human β globin chain (Fig 7, lanes 3 and 7). Translation of the β° -39 thalassemic mRNA in the presence of [^{35} S]-methionine, as expected, failed to produce any labeled peptide (Fig 7, lane 4), since the β globin chain contains a single methionine residue at amino acid 55, carboxy-terminal to the β° -39 thalassemia mutation.

Interestingly, however, the β° -39 thalassemic mRNA was able to produce a labeled peptide when incubated in the presence of [^{3} H]-leucine (Fig 7, lane 9). The translation product of β° -39 mRNA migrated faster than did normal β chains on Triton-acid-urea gels. It is tempting to speculate that the abnormally migrating β° -39 peptide is a β globin chain terminated by the translation stop codon at amino acid 39. The amount of material generated by cell-free translation precluded complete characterization of the abnormal peptide, however. The production of a labeled peptide by β° -39 thalassemic mRNA indicates that this message can interact with the translational apparatus and suggests that the β° -39 transcript may initiate polysome formation in vivo.

Splicing of normal β and β° -39 thalassemic pre-mRNAs. CAPped normal β globin and β° -39 thalassemic pre-mRNAs were spliced essentially as described by Krainer and co-workers²¹ in a HeLa cell nuclear extract,¹⁴ and the splicing products were separated on a denaturing 5% polyacrylamide gel (Fig 8). The intensities of the bands representing spliced exon 1 and 2, as well as exon 1 (cleaved but not spliced), from both normal β globin and β° -39 thalassemic pre-mRNAs are virtually equal, indicating that both pre-mRNAs are spliced to the same extent within 4 hours. The mutation at codon 39 near the 5' end of exon 2 in β° -39

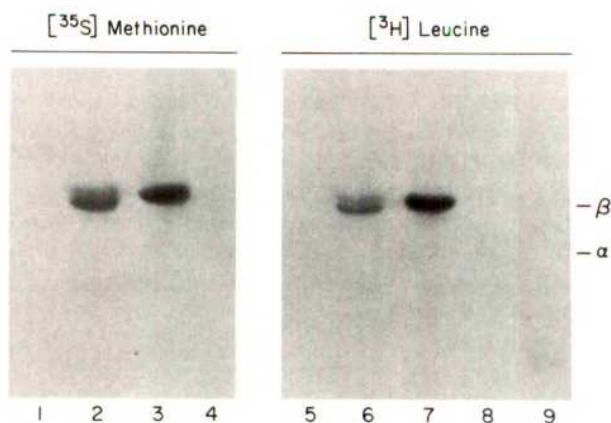


Fig 7. Translation of normal and β° -39 globin mRNA in a rabbit reticulocyte lysate. CAPped normal and β° -39 mRNA transcripts prepared from SP6 templates were translated in a rabbit reticulocyte in vitro translation system and labeled with [^{35} S]-methionine or [^{3} H]-leucine. The translation products were mixed with unlabeled adult human hemoglobin standards and electrophoresed on a 12.5% polyacrylamide gel containing acetic acid, urea, and Triton X-100. The gel was stained with Coomassie blue, processed for fluorography, dried, and exposed to x-ray film. Lanes 1 and 5, no mRNA; lanes 2 and 6, rabbit globin mRNA; lanes 3 and 7, normal β globin mRNA; lanes 4 and 8, β° -39 thalassemic mRNA; lane 9, longer exposure of lane 8. The mobilities of human α and β globin chain standards are noted (right).

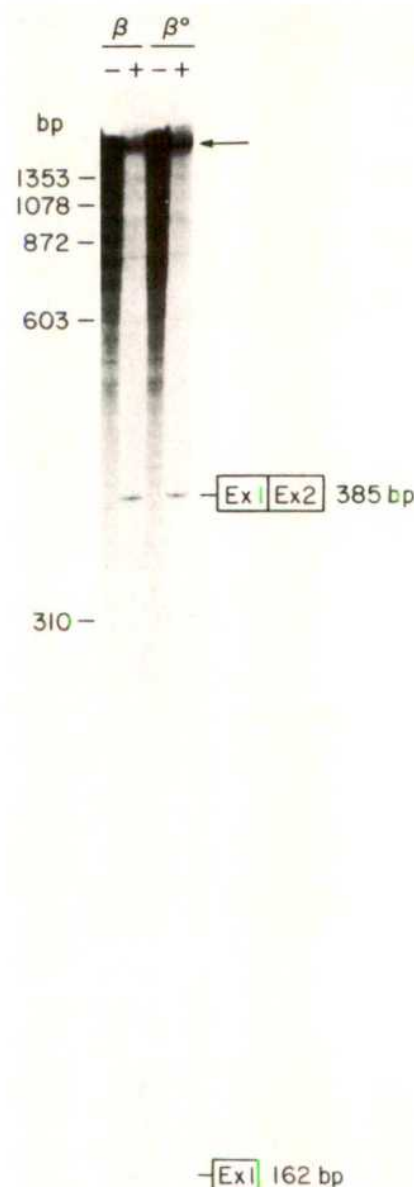


Fig 8. Splicing of normal and β° -39 globin pre-mRNA. CAPped normal β globin (β) and β° -39 thalassemic (β°) pre-mRNAs were electrophoresed on a denaturing 5% polyacrylamide gel before (–) or after (+) incubation in HeLa cell nuclear extract essentially as described by Krainer and colleagues.²¹ Position of RNA size markers is indicated (left). – Ex1 Ex2, product of splicing exons 1 and 2; – Ex1, fragment resulting from cleavage of exon 1 without splicing to exon 2. Arrow indicates position of unspliced pre-mRNA.

thalassemic pre-mRNA does not affect splicing of exons 1 and 2.

No appreciable splicing of exons 1 through 3 was observed in this reaction with either pre-mRNA. Humphries and colleagues,¹² however, and other researchers (S. Baserga and E.J. Benz, Jr, unpublished) have confirmed that β° -39 thalassemic pre-mRNAs in transfected cell lines are spliced as efficiently as normal β globin pre-mRNAs using an M13 probe covering exons 1, 2, and 3. Therefore, β° -39 thalas-

semic pre-mRNAs do not exhibit a defect in processing which might lead to their abnormal accumulation or degradation in the nucleus.

DISCUSSION

Decreased levels of β thalassemic mRNAs in the cell cytoplasm have been attributed either to degradation of nontranslatable message by ribonucleases in the cytoplasm,^{4,10} or to an intranuclear defect.^{11,12} Previous studies of the cytoplasmic stability of β^0 -39 transcripts in vivo and in transfected cells (using pulse-label and chase or actinomycin D chase techniques) indicated that the β^0 -39 mRNA was stable in the cytoplasm.^{11,12} It is difficult to draw firm conclusions from these results, however, because of the technical limitations inherent in the methods. The number of counts per minute that can be incorporated into globin mRNA in intact cells during a pulse-label is small; moreover, actinomycin D can affect other intracellular processes that can alter mRNA degradation rates.²² For these reasons, intact cell labeling studies cannot be used to distinguish short-term stability defects from transcription or processing phenomena. We have therefore examined the relative stabilities of normal β globin and β^0 -39 thalassemic transcripts using in vitro synthesized RNA transcripts and various cell-free extracts and, in particular, have addressed the question of whether translation of a β globin transcript affected its stability.

For this study, normal β globin and β^0 -39 thalassemic RNA transcripts were prepared which resembled authentic globin transcripts in that they could be CAPped, bound to oligo dT columns (not shown), translated, and spliced. The cell-free extracts chosen for study were ones previously developed to study discrete steps in RNA metabolism, such as translation, transcription, or splicing. Recently, Ross and Kobs²³ demonstrated the usefulness of such cell-free systems for detecting differences in stability of various mRNAs. In our cell-free systems, we found no difference in the relative stabilities of normal β or β^0 -39 thalassemic pre-mRNAs or mRNAs regardless of the translational potential of the extract or translatability of the RNA transcript.

The various cell-free extracts we examined exhibited quantitative and qualitative differences in ribonuclease activity. The rabbit reticulocyte lysate exhibited relatively little ribonuclease activity, consistent with its function as a translation extract. Some degradation did occur, however,

suggesting that the stability we observed was not merely the result of the EGTA used in the preparation of the lysate to inhibit micrococcal nuclease. Incubation in the rabbit reticulocyte lysate resulted in equal survival of both CAPped and non-CAPped transcripts. The HeLa whole cell extract, however, was capable of degrading non-CAPped transcripts. The 5' CAP structure is believed to protect CAPped transcripts from a 5' to 3' exoribonuclease,²⁴⁻²⁶ which is apparently the major ribonuclease activity in the HeLa cell extract. The MEL cell cytoplasmic extract, in contrast, was capable of degrading both CAPped and non-CAPped transcripts. The inability of the 5' CAP structure to protect transcripts in the MEL cell extract suggests that this extract contains potent endoribonuclease or 3' to 5' exoribonuclease activity not present in the HeLa cell extract. The clipping action of an endoribonuclease as opposed to the processive action of a 5' to 3' exoribonuclease might account for the fact that degradation of labeled RNA transcripts in the MEL cell extract resulted in a high percentage of fragments still capable of binding to a DE-81 filter as compared with the HeLa cell extract. Despite the differences in ribonuclease activity inherent in each cell-free extract, however, no extract was capable of preferentially degrading β^0 -39 thalassemic transcripts under any conditions tested.

The results reported here suggest that the stability of β^0 -39 mRNA is not dependent on polysome formation and that thalassemic RNA transcripts can be stable in the absence of translation. The recent observation by Baserga and colleagues²⁷ that there were no differences in stability between normal β globin and β^0 -39 thalassemic mRNAs in transfected cells containing a temperature-sensitive RNA polymerase, on incubation of the cells at the nonpermissive temperature, is consistent with our findings. Taken together, our results and those of others^{11,12,27} suggest that the decreased levels of β^0 -39 mRNA in patients' erythroid cells is not due to decreased cytoplasmic stability, but rather to some more "proximal" process such as transcription, nuclear stability, or nuclear to cytoplasmic transport.

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Biosynthesis and Secretion of Functional Protein S by a Human Megakaryoblastic Cell Line (MEG-01)

By Michinori Ogura, Noboru Tanabe, Junji Nishioka, Koji Suzuki, and Hidehiko Saito

A human megakaryoblastic cell line (MEG-01) was investigated for the presence of protein S in culture medium and cell lysates using a specific enzyme-linked immunoassay (ELISA) and a functional assay. When 5×10^5 MEG-01 cells/mL was subcultured in RPMI 1640 medium with 10% fetal calf serum (FCS), the concentration of protein S antigen in the culture medium increased progressively with time from <8 ng/mL on day 0 to 105.6 ± 6.0 ng/mL on day 13. Vitamin K₂ ($1 \mu\text{g/mL}$) increased the production of functional protein S, whereas warfarin ($1 \mu\text{g/mL}$) profoundly decreased the quantity and the specific activity of secreted protein S. By an indirect immunofluorescent technique, protein S antigen was detected in both MEG-01 cells and human bone marrow megakaryocytes. Immunoblot analysis of culture medium revealed two distinct bands (mol wt

84,000 and 78,000) that are identical to the doublets of purified plasma protein S. De novo synthesis of protein S was demonstrated by the presence of specific immunoprecipitable radioactivity in the medium after 5 hours of labeling of the cells with [³⁵S]-methionine as a 84,000 mol wt protein. Plasma protein S levels of nine patients with severe aplastic anemia were not significantly different from those of normal controls. These results suggest that megakaryocytes produce functional protein S and contain the enzymes required for the carboxylation of selected glutamic acid residues, and that protein S synthesized by megakaryocytes does not represent a main source of plasma protein S.

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PROTEIN S is a vitamin K-dependent single chain plasma glycoprotein that is required for optimal expression of the anticoagulant activity of another vitamin K-dependent protein, activated protein C (APC).¹ The physiologic importance of protein S as an anticoagulant is clearly illustrated by the association of hereditary protein S deficiency and thrombosis.² Recent studies have shown that a human hepatoma cell line, and human and bovine endothelial cells synthesize and secrete protein S.³⁻⁵ Schwarz and colleagues⁶ identified protein S in α granules of human platelets, which suggests that the released protein S may bind to stimulated platelets and thereby promote and localize the anticoagulant activity of APC on the platelet surface. Although protein S was recently observed in human megakaryocytes by immunochemical staining,⁷ it is not known whether megakaryocytes can synthesize protein S, since megakaryocytes may acquire some α -granule proteins from plasma by endocytosis.⁸ In this article, we report that a well-characterized human megakaryoblastic cell line, MEG-01,⁹ synthesizes and secretes functional protein S, which can be modulated by vitamin K and warfarin. A preliminary part of this study has been published in abstract form.¹⁰

MATERIALS AND METHODS

Human protein S, monospecific rabbit antiserum against protein S, and citrated plasmas of 25 normal volunteers and nine patients with severe aplastic anemia were prepared as described.¹¹ A monospecific rabbit antiserum against von Willebrand factor (vWf) was obtained from Dakopatts, Grostrup, Denmark.

Cell culture. The establishment and several properties of a human megakaryoblastic cell line, MEG-01, have been previously described in detail.⁹ MEG-01 cells possessed no markers for B or T lymphocytes or for myeloid cells. They demonstrated many megakaryocytic characteristics.⁹ Additional studies showed that cell surface glycoprotein A was not demonstrated by an indirect immunofluorescent technique using mouse anti-human glycoprotein A monoclonal antibody (Immunotech, Marseille, France), indicating the absence of the erythroid antigen. MEG-01 cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS, Flow Laboratories, Stanmore, New South Wales, Australia), 100 U/mL aqueous penicillin G, and 50 $\mu\text{g/mL}$ streptomycin. In some experiments, serum-free medium (HB101, Hana

Media, Berkeley, CA) was also used. The cells were seeded in Falcon 3013 plastic tissue culture flasks (Falcon, Division of Becton Dickinson, Oxnard, CA) at 2 to $5 \times 10^5/\text{mL}$ in 8 mL of medium. In selected experiments, the cells were treated with vitamin K₂ (Kayt-wo, Eisai, Tokyo; $1 \mu\text{g/mL}$) or warfarin derivative (3-(α -acetylbenzyl)-4-hydroxycoumarin potassium salt, warfarin potassium, Eisai, $1 \mu\text{g/mL}$) for 7 to 14 days in RPMI 1640 medium with 10% FCS or in HB101 serum-free medium. Aliquots (1.8 mL) were removed daily or every 2 to 3 days, and the same volume of fresh medium was replaced. Harvested medium was mixed with 0.2 mL of phosphate-buffered saline, pH 7.0, (PBS) containing 500 mmol/L of benzamide hydrochloride (Sigma Chemical, St Louis) and 10 mmol/L of diisopropyl fluorophosphate (DFP, Sigma), and was centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatants were stored at -80°C until analysis. Cell pellets were suspended in cold PBS containing protease inhibitors described above and 0.1% Triton X-100 (Sigma) and frozen and thawed twice. Cells were further disrupted by an ultrasonic cell disruptor (Sonifier, model 200, New York), and were centrifuged at 15,000 rpm for 10 minutes. The supernatants of cell lysates were also frozen until use. Spent medium and cell lysates of other human cell lines such as HL-60 (promyelocytic leukemia cell line), K-562 (erythroleukemia cell line), MOLT 4 (T lymphoblastic leukemia cell line), and U-937 (histiocytic lymphoma cell line) were used as controls for MEG-01.

Assay of protein S antigen. Protein S was determined by the sandwich-type enzyme-linked immunoassay (ELISA) using mono-

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specific rabbit anti-protein S F(a,b')₂ fragment coupled with β -D-galactosidase that had been prepared as described.¹² Assays were performed at room temperature. Flat-bottomed ELISA plate wells were coated with the F(a,b')₂ fragment (10 μ g/mL) in 0.1 mol/L of phosphate buffer, pH 8.0. After 1 hour of incubation, unbound materials were removed by washing with the phosphate buffer. The wells were then blocked with 200 μ L of 10% normal rabbit serum for 2 hours. The wells were washed three times with PBS (0.01 mol/L of phosphate buffer, 0.1 mol/L of NaCl, 1 mmol/L of MgCl₂, pH 7.0) containing 0.1% bovine serum albumin (BSA). Then 100 μ L of sample solution, diluted appropriately in PBS containing 0.1% BSA, was added and incubated for 2 hours. After being washed three times, 100 μ L of the F(a,b')₂ fragment coupled with β -D-galactosidase was added and incubated for 1 hour. After being washed, 100 μ L of freshly prepared substrate solution, 0.1 mg/mL of 4-methyl-umbelliferyl- β -D-galactoside (Sigma) in the PBS, was then added to the wells, and the enzyme reaction was allowed to proceed for 30 minutes. Fluorescence of each well was determined at 360 nm excitation and 450 nm emission using Fluororeader (Dynatech Laboratories, Inc, Alexandria, VA). By this ELISA, protein S 1 to 100 ng/mL was measured with high specificity and reproducibility. Protein S antigen of plasmas from the patients with aplastic anemia and normal controls was measured by the Laurell electroimmunoassay as described.¹¹

Functional assay of protein S. The culture medium was concentrated 20-fold with Centricon (Amicon, Danvers, MA), and then dialyzed against 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.5. Preliminary experiments showed that 97% protein S was recovered during concentration and dialysis when known amounts of purified protein S were added to the culture medium before concentration. Protein S activity was assayed by determining the ability of protein S to function as a cofactor of the anticoagulant effects of activated protein C as described earlier.¹¹ The standard curve was prepared by substituting the culture medium with protein S-depleted plasma containing varying amounts of purified protein S (0, 0.31, 0.63, 1.25, 2.5, 5, 10 μ g/mL). The fresh culture medium with 10% FCS contained no significant amount of protein S activity.

Immunoblotting analysis of protein S in culture medium. Culture medium (35 mL) was mixed with 2 mL of the agarose coupled with anti-protein S-IgG (2 mg of IgG/mL of agarose) at room temperature for 4 hours in a rolling bottle. The agarose was then packed in a column (0.9 $\times$ 6 cm) and washed with 20 mL of 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.5. Proteins bound to the agarose were then eluted with 0.05 mol/L of Tris-HCl, pH 7.5, containing 8 mol/L of urea. Protein S in the eluate was examined with sodium dodecyl sulfate (SDS)-polyacrylamide gradient (7.5% to 15%) slab gel electrophoresis¹³ in the presence of a reducing agent, followed by the Western blotting technique using anti-protein S-serum, as previously described.¹⁴

Immunoprecipitation. Incorporation of [³⁵S]-methionine into protein S was studied as described.¹⁵ In brief, cultures of MEG-01 cells were washed with methionine-free minimal essential medium (Nissui Seiyaku, Tokyo) and were then incubated in 5 mL of the same medium supplemented with 1 mCi [³⁵S]-methionine (Amersham, Arlington Heights, IL) for 5 hours at 37°C. Protein S was immunoprecipitated from the medium by the method of Kessler.¹⁶ The immunoprecipitates were eluted from formaldehyde-fixed *Staphylococcus aureus* Cowan strain II (Pansorbin, Calbiochem-Behring, La Jolla, CA) by boiling and then electrophoresed on SDS-9% polyacrylamide gel electrophoresis (PAGE). Gels were stained, destained, and then treated with Enlightening (New England Nuclear, Boston) before autoradiography.

Immunofluorescence staining. Bone marrow specimens were obtained from patients with nonmyeloproliferative disorders after informed consent. Heparinized bone marrow cells were resuspended

in ~10 vol of erythrocyte-lysing buffer (155 mmol/L of NH₄Cl, 10 mmol/L of KHCO₃, and 2.5 mmol/L of EDTA, pH 7.3, 37°C) and immediately centrifuged. The marrow cells were resuspended and washed by centrifugation three times in PBS supplemented with 0.75% BSA and 2.5 mmol/L of EDTA. Washed marrow cells and MEG-01 cells were analyzed for the presence of vWf and protein S antigen in cytoplasm by an indirect immunofluorescent technique as described.⁹ Other human hematopoietic cell lines described above were also examined for the presence of cytoplasmic protein S.

RESULTS

Appearance of protein S in the culture medium. When 5×10^5 /mL of MEG-01 cells was subcultured in the new medium supplemented with 10% FCS, the concentration of protein S antigen in the medium increased progressively with time from <8 ng/mL on day 0 to 105.6 ± 6.0 ng/mL on day 13 (Fig 1). Each point in Fig 1 represents the mean $\pm$ SD of triplicate flasks. A similar increase was also found in the experiments using HB101 serum-free medium (data not shown). Cell lysates quantitatively analyzed for protein S antigen gave no evidence for a large intracellular pool of this protein. Immunoblot analysis of spent medium (day 2) revealed two distinct bands of mol wt ~84,000 and ~78,000 (Fig 2, lane 2) that are identical to the doublets of purified plasma protein S (lane 1). A faint band just below 78,000 band in lane 2 is probably a degradation product of protein S. In contrast, culture medium and cell lysates (day 5) of HL-60, K-562, MOLT 4 and U-937 cells contained no significant amount of protein S antigen (<2.5 ng/mL).

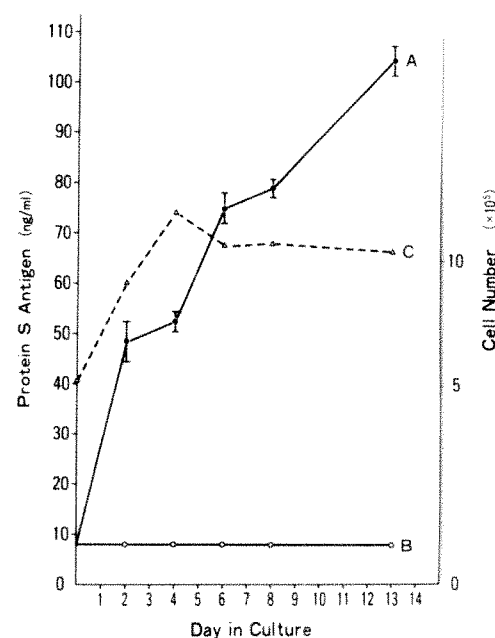


Fig 1. Time course of the accumulation of protein S antigen in the culture medium (A) and cell lysates (B) of MEG-01 cells. Three Falcon 3013 flasks were each seeded with 5×10^5 /mL MEG-01 cells in 8 mL of RPMI 1640 medium containing 10% fetal calf serum (FCS). On the days indicated, 1.8 mL of medium was removed and replaced with fresh medium. Antigen was measured using a specific enzyme-linked immunoassay (ELISA). The values are plotted as the mean $\pm$ SD. Line (C) shows the cell growth curve.

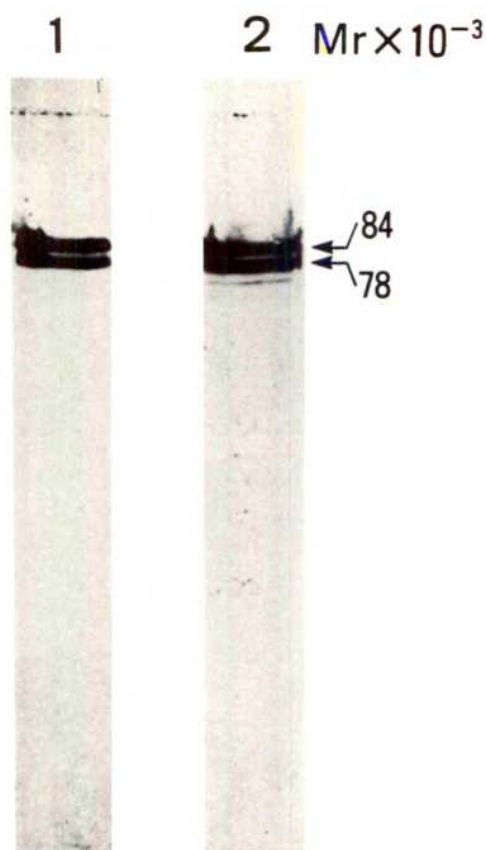


Fig 2. Immunoblot of purified protein S (lane 1) and day 3 culture medium (lane 2) shows the same doublets of mol wt 84,000 and 78,000. A faint band just below 78,000 is probably a degradation product of protein S.

Effect of vitamin K and warfarin. Because protein S is a vitamin K-dependent protein, the effects of vitamin K₂ and warfarin on the quantity and activity of secreted protein S by MEG-01 cells were examined. Vitamin K₂ (1 µg/mL) enhanced the accumulation of secreted protein S antigen in the medium, whereas warfarin (1 µg/mL) inhibited the secretion of this protein. Similar effects of vitamin K₂ and warfarin were also observed in the experiments with HB101 serum-free medium (data not shown).

Table 1. Effect of Vitamin K₂ and Warfarin on the Activity and Antigen of Secreted Protein S by MEG-01

	Protein S		Activity
	Antigen (ng/mL)	Activity (ng/mL)	Antigen
Fresh medium	5	0	0
Control culture	440	380	0.86
V-K ₂ in culture	680	750	1.10
Warfarin in culture	280	15	0.05

MEG-01 cells were seeded in Falcon 3013 flasks at 2×10^5 /mL in 40 mL of RPMI 1640 medium containing 10% fetal calf serum and cultured for 5 days with or without vitamin K₂ (1 µg/mL) and warfarin (1 µg/mL). Culture media, after 20-fold concentration, were assayed for protein S antigen and activity as described in the Materials and Methods section. Purified plasma protein S was used as a standard in both assays, and the results were expressed as nanogram of protein S per milliliter of 20-fold concentrated medium.

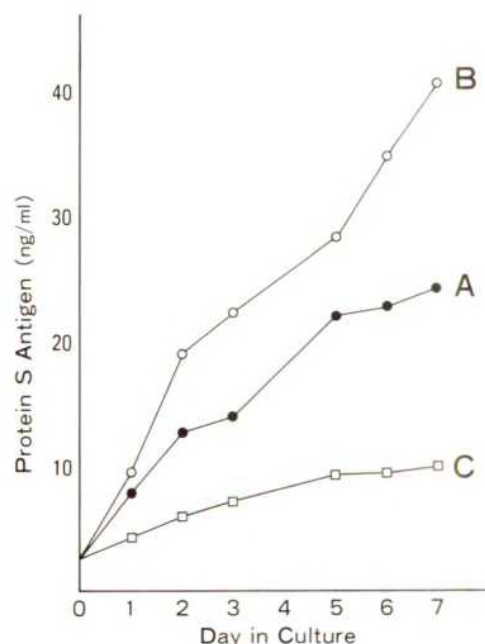


Fig 3. Time course of the accumulation of protein S antigen in the control culture medium (A), culture medium with 1 µg/mL of vitamin K₂ (B) and culture medium with 1 µg/mL of warfarin (C). MEG-01 cells were seeded at 2×10^5 /mL in 8 mL of RPMI 1640 medium containing 10% fetal calf serum (FCS). Values are expressed as nanogram of protein S antigen per milliliter of medium.

Table 1 shows the activity and antigen of secreted protein S when MEG-01 cells were cultured for 5 days in the absence or presence of vitamin K₂ and warfarin. As judged by the ratio (0.86) of activity/antigen, control MEG-01 cells appear to secrete fully functional protein S. Vitamin K₂ enhanced the secretion of functional protein S (activity/antigen: 1.10), whereas warfarin decreased both quantity and specific activity (0.05) of secreted protein S.

Incorporation of [³⁵S]-methionine into immunoprecipitable protein. To demonstrate de novo synthesis of protein S, we cultured the cells in the presence of [³⁵S]-methionine for 5 hours and immunoprecipitated the culture medium with anti-protein S serum. SDS-PAGE demonstrated a single band of mol wt ~84,000 (Fig 4, lane 1) that was absent in the control immunoprecipitates (Fig 4, lane 2). These data clearly indicate that MEG-01 cells synthesize and secrete protein S.

Immunofluorescence stainings. MEG-01 cells and bone marrow megakaryocytes showed positive granular staining patterns with anti-protein S serum (Figs 5 and 6). No other nucleated cells in bone marrow except megakaryocytes or HL-60, K 562, MOLT 4, and U-937 cells were positively stained. No positive staining was observed when MEG-01 cells and megakaryocytes were first reacted with normal rabbit serum and then with fluorescein isothiocyanate-conjugated second antibody. The pattern of staining of megakaryocytes or MEG-01 cells with anti-protein S serum was similar to that with anti-vWf serum, suggesting the presence of protein S in the cytoplasm of MEG-01 cells.

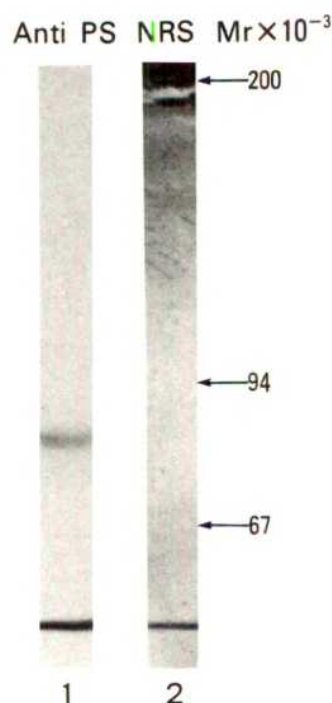


Fig 4. Autoradiogram of the immunoprecipitates from [^{35} S]-methionine-labeled proteins secreted from MEG-01 cells following incubation with normal rabbit serum (lane 2) and anti-protein S serum (lane 1).

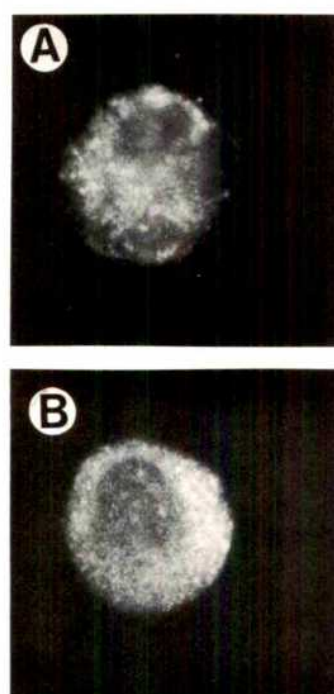


Fig 5. Immunofluorescent staining of human megakaryocytes from bone marrow. Staining with anti-von Willebrand factor serum (A) reveals a granular staining pattern; staining with anti-protein S serum (B). (Original magnification $\times 400$.)

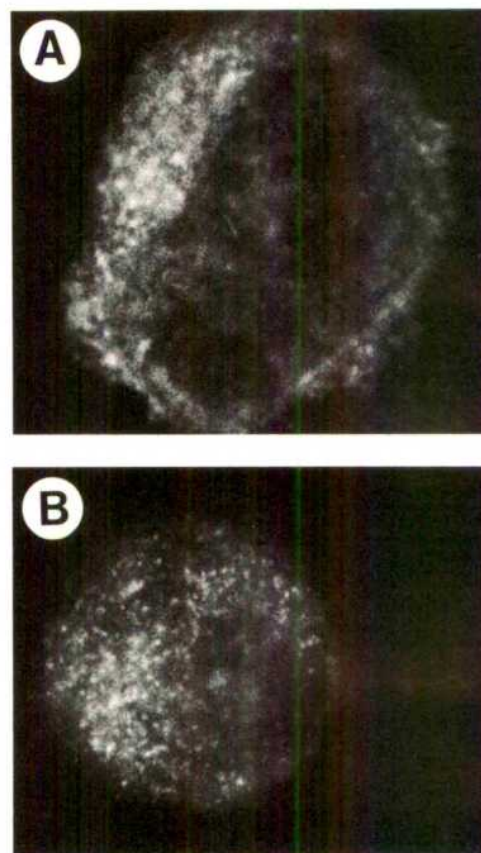


Fig 6. Immunofluorescent staining of MEG-01 cells. Staining with anti-von Willebrand factor serum (A); staining with anti-protein S serum (B). Granular staining pattern is observed in both photographs. (Original magnification $\times 1,000$; current magnification $\times 700$.)

Plasma protein S antigen in patients with aplastic anemia. The mean $\pm$ SD of protein S antigen in nine patients with untreated severe aplastic anemia was $26.9 \pm 2.8 \mu\text{g/mL}$, whereas that in 25 normal volunteers was $26.2 \pm 4.5 \mu\text{g/mL}$.

DISCUSSION

It was previously simply assumed that synthesis of vitamin K-dependent blood clotting factors was restricted to hepatocytes. Recent studies, however, have demonstrated that human monocyte-macrophage cells synthesize factor VII^{17,18} and that human and bovine endothelial cells produce protein S.^{4,5} Thus, other nonhepatocyte cells may also participate in the biosynthesis of some vitamin K-dependent coagulation proteins. Human platelets contain protein S,⁶ and the presence of this protein in human megakaryocytes and a human erythroleukemia cell line (HEL) has recently been reported.⁷ These studies suggested, but did not prove, that megakaryocytes may synthesize protein S.

In this report, we have provided definitive evidence for the production of functional protein S by human megakaryocytes. A human megakaryoblastic cell line (MEG-01) has

the capacity to synthesize and secrete functional protein S, which has a mol wt similar to that of the plasma form.¹⁹ Its synthesis can be affected by known pharmacologic modulators of vitamin K-dependent proteins. Protein S was detected in both MEG-01 cells and human megakaryocytes by indirect immunofluorescent technique. Although the immunohistochemical staining revealed protein S in the cytoplasm, MEG-01 cells appear to have a very small intracellular pool of this protein (Fig 1). MEG-01 cells are megakaryoblasts that lack α granules⁹; therefore, protein S synthesized by the cells may not be retained in the cytoplasm but rapidly secreted. β -Thromboglobulin, another platelet α -granule protein, was also synthesized and rapidly secreted as protein S (M.O., N.T., T.H., unpublished observations). Because MEG-01 cells are derived from blast crisis of chronic myelogenous leukemia (CML), it may be difficult to decide if the production of protein S by these cells reflects synthesis of normal megakaryocyte gene product or arises from gene depression due to transformation. MEG-01 cells, however, appear to retain the morphological features and the differentiated functions of normal megakaryoblasts.⁹ Leukemic buffy coat cells from a similar patient with accelerated phase of CML were also used as a model to demonstrate biosynthesis of von Willebrand protein by human megakaryocytes.²⁰

The observation that vitamin K₂ enhanced and warfarin inhibited the secretion of functional protein S by MEG-01 cells is similar to that of the secretion of protein S or

prothrombin by a human hepatoma cell line (Hep G2)^{3,21} or the protein S secretion by human and bovine endothelial cells.^{4,5} These results suggest that MEG-01 cells contain the enzymes required for γ -carboxylation.

Human platelets have been reported to contain 2.5% of protein S in whole blood.⁶ Because in the present study we found that megakaryocytes secrete protein S, we evaluated the contribution of megakaryocytes to the plasma level of this protein. Plasma protein S exists in two forms; free protein S and C4b-binding protein-protein S complex.²² The electroimmunoassay used in this study measures both free and complexed protein S (total protein S). There was no significant difference between the total protein S levels of small numbers of patients with severe aplastic anemia and those of normal controls. Thus, it is unlikely that protein S synthesized by megakaryocytes represents a main source of plasma protein S.

The present study adds protein S to a list of coagulation proteins such as vWf, factor V, and fibrinogen, which are synthesized by human megakaryocytes. The role of protein S produced by megakaryocyte in the local control of hemostasis and thrombosis remains to be investigated.

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Detection of Activated Platelets in Whole Blood Using Activation-Dependent Monoclonal Antibodies and Flow Cytometry

By Sanford J. Shattil, Michael Cunningham, and James A. Hoxie

Platelets may become activated in a number of clinical disorders and participate in thrombus formation. We developed a direct test for activated platelets in whole blood using flow cytometry. Whole blood was incubated with either biotin-PAC1, a monoclonal antibody specific for the fibrinogen receptor on activated platelets, or biotin-S12, an antibody specific for an α -granule membrane protein that associates with the platelet surface during secretion. Platelet-bound antibodies were detected with streptavidin conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Platelets were differentiated from the larger erythrocytes and WBCs by their light-scatter profile. Alternatively, platelets could be identified with FITC-AP1, an antibody specific for platelet membrane glycoprotein Ib, and analyzed further for PAC1 or S12 binding with PE-streptavidin. No centrifugation or washing steps were required. With gel-filtered platelets, there was a direct correlation between ADP-induced biotin-PAC1 binding and binding determined in a conventional ^{125}I -PAC1 binding assay ($r = .99$; $P < .001$). Furthermore, as few as 0.8% activated platelets could be detected by flow cytometry

when activated platelets were mixed with unstimulated platelets. In whole blood, unstimulated platelets demonstrated no PAC1- or S12-specific fluorescence, indicating that they did not bind these antibodies. On stimulation with agonists, however, the platelets demonstrated a dose-dependent increase in fluorescence similar to that observed for platelets in plasma or buffer. Low concentrations of ADP and epinephrine, which induce fibrinogen receptors but little secretion, stimulated near-maximal PAC1 binding but little S12 binding. On the other hand, a concentration of phorbol myristate acetate (TPA) that evokes full platelet aggregation and secretion induced maximal PAC1 and S12 binding. Activated platelets could also be analyzed in whole blood samples that had been fixed with paraformaldehyde. These studies demonstrate that activated platelets can be reliably detected in whole blood using activation-dependent monoclonal antibodies and flow cytometry. This technique may be useful to assess the degree of platelet activation and the efficacy of antiplatelet therapy in clinical disorders.

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CHANGES OCCUR at the platelet surface during cell activation. First, agonists bind to their membrane receptors and initiate the activation process. Then, for platelets to aggregate, heterodimer complexes of the membrane glycoproteins IIb and IIIa must be converted into competent receptors for fibrinogen.¹ During platelet secretion, granule membranes fuse with the plasma membrane or with membranes of the surface-connected open canalicular system.² The metabolic reactions responsible for these surface changes are only partly understood. In response to some agonists, such as thrombin, the exposure of fibrinogen receptors and the secretory response can occur as a result of agonist-induced phosphoinositide hydrolysis.³⁻⁸ On the other hand, weaker agonists such as epinephrine can stimulate fibrinogen receptor expression independent of phosphoinositide hydrolysis and cause secretion by stimulating arachidonic acid metabolism and thromboxane A_2 synthesis.⁹⁻¹¹

Recently, several murine monoclonal antibodies have been described that recognize antigenic determinants on the platelet surface, but only after platelet activation.^{9,12-14} For example, PAC1 is an IgM antibody that binds only to the activated form of the IIb-IIIa complex.⁹ It appears to recognize an epitope on the IIb-IIIa complex critical for fibrinogen binding. PAC1 and fibrinogen inhibit each other's binding in a competitive manner.^{9,15} Moreover, PAC1 binding is competitively inhibited by the tetrapeptide, Arg-Gly-Asp-Ser, which is present in the α chain of fibrinogen and functions as a cell attachment site for fibrinogen and other adhesive macromolecules.¹⁶ PAC1 has been useful in identifying the intracellular mediators responsible for fibrinogen receptor expression in activated platelets.³ Two other "activation-dependent" antibodies, S12 and KC4, recognize a 140-kd α -granule membrane protein that becomes associated with the platelet surface during secretion.^{12,13,17,18} Radiolabeled S12 has been used to detect activated platelets

in blood samples from patients with acute respiratory distress syndrome.¹⁹ Thus, activation-specific monoclonal antibodies may be useful both in unraveling the biochemical mechanisms of platelet activation and in detecting activated platelets in clinical disorders.

When platelets are stimulated to undergo a secretory response, they produce thromboxane A_2 and release the contents of their storage granules into the extracellular milieu. Some of these released substances, such as platelet factor 4 and β -thromboglobulin, are platelet-specific and can be detected in plasma by sensitive radioimmunoassays.²⁰ The measurement of these proteins in plasma and of thromboxane A_2 metabolites in plasma and urine has provided evidence for platelet activation in several clinical disorders.^{21,22} Although helpful in a research setting, these indirect methods have technical limitations that have precluded

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their routine clinical use. For example, extreme care must be taken during blood sample collection and processing to prevent the *in vitro* release of platelet factor 4 and β -thromboglobulin.^{20,21}

The purpose of the present study was to develop a sensitive and specific direct assay for activated platelets in whole blood. To accomplish this, we used the activation-specific monoclonal antibodies, PAC1 and S12. The binding of these antibodies to platelets was detected using the sensitive technique of flow cytometry, which is capable of detecting and quantitating antigens on the surface of individual platelets.²³⁻²⁶

METHODS

Preparation of Monoclonal Antibodies

PAC1, an IgM murine monoclonal antibody specific for the activated form of the platelet membrane glycoprotein IIb-IIIa complex, and A₂A₆, an IgG antibody specific for the IIb-IIIa complex on resting or activated platelets, were purified as described previously.⁹ AP1, an IgG antibody specific for membrane glycoprotein Ib,²⁷ was kindly provided by Dr Thomas Kunicki, Blood Center of Southeastern Wisconsin, and purified by sequential precipitation of non-IgG proteins using rivanol and caprylic acid (T. Kunicki, personal communication). S12, an IgG antibody specific for a 140-kD α -granule membrane protein,^{13,18} was generously provided in pure form by Dr Rodger McEver, University of Texas at San Antonio. Antibody preparations were pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Prior to their use in flow cytometry, these antibodies were conjugated either with biotin or with fluorescein isothiocyanate (FITC). Antibodies were biotinylated by incubating 500 μ g of antibody in 500 μ L of phosphate-buffered saline (PBS), pH 7.4, for 2 hours at room temperature with 50 μ L of 3.2 mmol/L of *N*-hydroxysuccinimide biotin ester in dimethyl sulfoxide and 50 μ L of 1 mol/L of NaHCO₃. The reaction was stopped with 50 μ L of 1 mol/L of NH₄Cl for 10 minutes, and the mixture was passed over a Sephadex G-25 column equilibrated with PBS. The biotinylated protein appeared in the void volume and was stored at 4°C until use. Antibodies were conjugated to FITC by first adjusting the pH of the antibody solution (1 mg/mL) to 8.5 to 9 with one-tenth vol of 1 mol/L of NaCO₃. Then, 1 mg of FITC-Celite (Calbiochem, San Diego) was added per milligram of antibody and the mixture was incubated for 10 minutes at room temperature with intermittent shaking. The sample was applied to a Sephadex G-25 column equilibrated with PBS, and FITC-conjugated antibody was recovered in the void volume. The fluorescein/protein molar ratio ranged between 3 and 4 for AP1 and S12 and 12 and 15 for PAC1. Biotin- and FITC-conjugated antibodies appeared to function normally in that their binding to platelets was saturable at antibody concentrations reported for the unconjugated species.^{9,13,27} In some experiments, PAC1 was radiolabeled with ¹²⁵I to compare the binding of the radiolabeled form of the antibody⁹ with that of the biotinylated form.

Preparation of Whole Blood Samples for Flow Cytometric Analysis

Blood was obtained from normal volunteers who had taken no medications for at least 10 days. In an attempt to minimize platelet activation during blood collection, the donor was supine, and blood was obtained from an antecubital vein through a 19-gauge butterfly needle with either a light tourniquet or no tourniquet. After the first

2 mL of blood were discarded, 4.5 mL of blood was collected into a plastic syringe containing 0.5 mL of 3.8% sodium citrate. In some experiments, the platelets were then fixed with 1% paraformaldehyde as described by George and colleagues.¹⁹ Within 1 minute of the collection of fresh blood or 45 minutes after platelet fixation, 5- μ L aliquots of the blood (containing 0.75 to 1.0 $\times$ 10⁶ platelets) were added to 12 $\times$ 75-mm polystyrene tubes containing 50 μ L of an isotonic HEPES buffer⁹ and 5 μ L of a saturating concentration of biotin-PAC1 (30 μ g/mL) or biotin-S12 (10 μ g/mL). In some experiments, platelets were activated by including 5 μ L of epinephrine, ADP, or phorbol myristate acetate (PMA) in the tubes. The samples were incubated at room temperature for 15 minutes without stirring. As described in detail below, platelets were analyzed by flow cytometry using either a single fluorochrome (the "one-color" method) or two fluorochromes (the "two-color" method). For the one-color method, after the incubation with the antibodies, a saturating concentration of FITC-streptavidin (5 μ L of a 1:10 dilution; Amersham, Arlington Heights, IL) was added, and the tubes were incubated for an additional 15 minutes. The samples were then diluted with 500 μ L of the isotonic buffer and analyzed. No washing steps were required. For the two-color method, one fluorescent reagent, FITC-AP1, was used to label and identify all the platelets while a second fluorescent reagent, phycoerythrin (PE)-streptavidin (Becton Dickinson, Mountain View, CA) was used to detect biotin-PAC1 or biotin-S12 bound to activated platelets. Specifically, after 15 minutes of incubation with the biotinylated antibody, 15 μ L of PE-streptavidin and 2 μ L FITC-AP1 was added for 15 minutes, and the sample was diluted and analyzed. In some studies, platelet-rich plasma (PRP) or gel-filtered platelets were prepared⁹ and then examined by flow cytometry as described above for whole blood.

Flow Cytometric Analysis

Blood samples were analyzed in a Becton Dickinson FACStar flow cytometer. The instrument was equipped with a 5 W argon laser and operated at 200 mW power at a wave length of 488 nm. Fluorescein fluorescence was detected using a 530/30 band pass filter, and PE fluorescence was detected with a 585/42 filter. The instrument was calibrated for fluorescence and light scatter daily using 2- μ m Calibrite beads (Becton Dickinson). Blood samples were passed through the laser beam through a 70- μ m nozzle at a flow rate of 10,000 blood cells per second. Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode, and the data were analyzed on a Hewlett-Packard Consort 30 H-P 217 computer (Palo Alto, CA).

For one-color analysis, the platelets were distinguished from erythrocytes and WBCs on the basis of their forward- and side-light scatter profile (described in the Results section). Debris or "machine noise" demonstrated a scatter profile distinctly smaller than that of platelets and was excluded from the analysis by setting the appropriate forward-scatter threshold. A gate was set around the platelets, and 10,000 cells were analyzed for FITC fluorescence to quantitate the amount of platelet-bound PAC1 or S12. Antibody binding was expressed either as the mean fluorescence intensity or as the percentage of platelets positive for antibody. Antibody-positive cells were defined as those platelets with a fluorescence intensity >99.0% to 99.5% of unstimulated platelets that had been prepared in the presence of 1 μ mol/L of PGI₂.

For two-color analysis, a fluorescence threshold was set to analyze only those blood cells that had bound FITC-AP1. Because erythrocytes and WBCs do not bind this platelet-specific antibody, they were effectively excluded from the analysis. Platelet-bound biotin-PAC1 or S12 was then determined by analyzing 10,000 platelets for PE-streptavidin fluorescence.

RESULTS

Detection of Activated Gel-Filtered Platelets With Monoclonal Antibodies

To explore the feasibility of using activation-dependent monoclonal antibodies to detect activated platelets by flow cytometry, initial studies were performed with gel-filtered platelets. For these studies, the one-color method of flow cytometric analysis, described in the Materials and Methods section, was used. The binding of biotin-PAC1 as assessed by flow cytometry was compared with the binding of ^{125} I-PAC1 determined in a standard filtration binding assay. Gel-filtered platelets were incubated with PGI_2 to prevent platelet activation or were stimulated with concentrations of ADP ranging from 0.01 to 5 $\mu\text{mol/L}$. Whether biotin-PAC1 binding was expressed as mean fluorescence intensity or as the percentage of platelets binding PAC1, there was a high correlation between binding detected by flow cytometry and that detected with ^{125} I-PAC1 ($r = .99$; $P < .001$) (Fig 1). An advantage of flow cytometry over conventional binding assays is its ability to detect heterogeneity in platelet responsiveness to agonists. When platelets were incubated with biotin-PAC1 in the presence of 0.4 $\mu\text{mol/L}$ of ADP, only a portion of the platelets demonstrated fluorescence above baseline (Fig 2). With a higher concentration of ADP (4 $\mu\text{mol/L}$), an increasing percentage of the platelets demonstrated positive fluorescence, although these cells varied greatly in the extent to which they had bound PAC1. At an even higher concentration of ADP (40 $\mu\text{mol/L}$), most platelets had bound maximal or near-maximal amounts of PAC1.

To see if flow cytometry could detect minor degrees of platelet activation, platelets were activated with 20 $\mu\text{mol/L}$ of ADP and epinephrine and labeled with biotin-PAC1. These platelets were then mixed with various amounts of unactivated platelets that had been labeled with PAC1 in the presence of PGI_2 . Even when the final platelet mixture

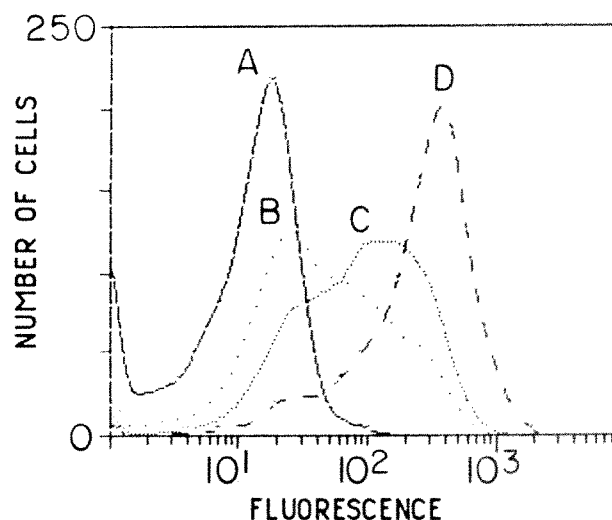


Fig 2. Identification of subpopulations of activated platelets by flow cytometry. Gel-filtered platelets were incubated for 15 minutes with biotin-PAC1 in the presence of no ADP (A), 0.4 $\mu\text{mol/L}$ of ADP (B), 4 $\mu\text{mol/L}$ of ADP (C) or 40 $\mu\text{mol/L}$ of ADP (D). Then FITC-streptavidin was added, and the cells were analyzed for PAC1 binding by flow cytometry.

contained as few as 0.8% activated platelets, these could still be detected and differentiated from a control sample containing only unactivated platelets (Fig 3).

Fibrinogen is a competitive inhibitor of PAC1 binding to platelets.^{9,15} Because this is a potential problem with whole blood, in which plasma fibrinogen is present in large amounts, biotin-PAC1 binding to platelets was compared in plasma and buffer. When a saturating concentration of PAC1 was added simultaneously with an agonist such as ADP or phorbol ester, there was no difference in PAC1 binding between PRP and gel-filtered platelets over a range of agonist concentrations (not shown). This may be due to

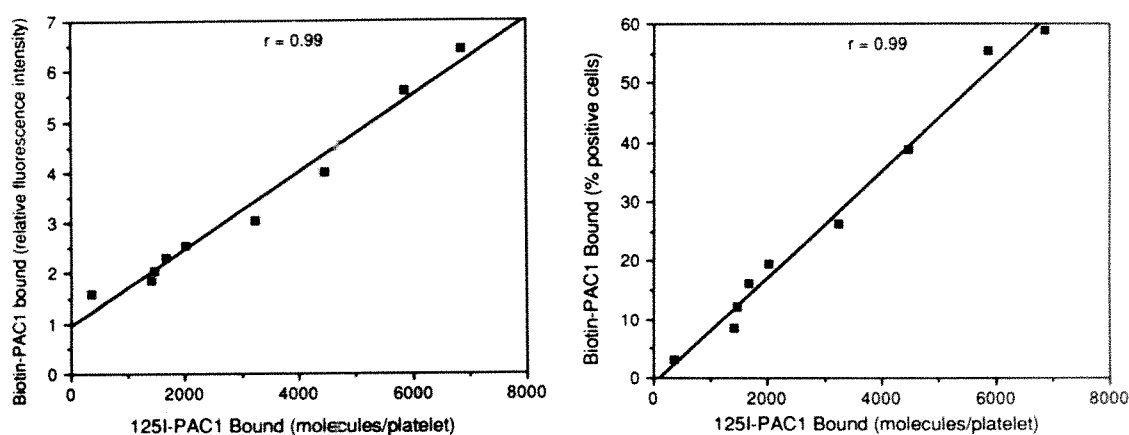


Fig 1. Relationship between biotin-PAC1 binding and ^{125} I-PAC1 binding to ADP-stimulated platelets. Gel-filtered platelets were incubated either with 1 $\mu\text{mol/L}$ PGI_2 to prevent platelet activation, or stimulated for 1 minute with various concentrations of ADP ranging from 0.01 to 5.0 $\mu\text{mol/L}$. They were then incubated with ^{125} I-PAC1 (18 $\mu\text{g/mL}$), and the amount of PAC1 bound to the platelets was determined. In parallel, the platelets were incubated with biotin-PAC1, and PAC1 binding was quantitated by flow cytometry. Biotin-PAC1 binding was expressed either as the mean fluorescence intensity of stimulated platelets relative to PGI_2 -treated platelets (left panel) or as the percentage of cells exhibiting positive fluorescence (right panel). Data represent the mean of two experiments.

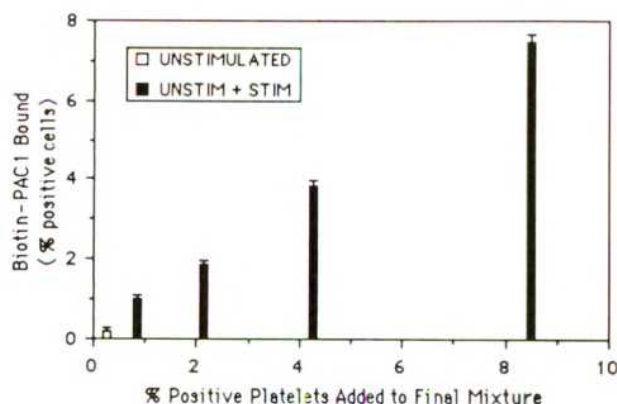


Fig 3. Detection of activated platelets when mixed with unstimulated platelets. Gel-filtered platelets were stimulated with a combination of 20 $\mu\text{mol/L}$ of ADP and epinephrine and then incubated with biotin-PAC1. The sample contained an average of 85% PAC1-positive platelets. Small but increasing amounts of the sample were then added to a sample of unstimulated, PGI_2 -treated platelets, and the final mixtures were analyzed for PAC1 binding by flow cytometry. The scale on the ordinate ranges from 0% to 10% positive platelets. All mixtures containing stimulated platelets showed more PAC1-positive cells than did the PGI_2 control ($P \leq .01$). Data represent the mean $\pm$ SEM of four experiments.

the greater apparent affinity of PAC1 ($K_d = 5 \text{ nmol/L}$) than fibrinogen ($K_d = 250 \text{ nmol/L}$) for the fibrinogen receptor.^{9,15} Another potential confounding effect of fibrinogen in whole blood assays is that the binding of this ligand is initially reversible but becomes progressively irreversible over the course of 60 minutes.¹ Indeed, when biotin-PAC1 was added to PRP after stimulation of the platelets with an agonist, there was a time-dependent reduction in the mean fluorescence intensity of PAC1-positive cells, suggesting that fibrinogen had become irreversibly bound to a portion of the fibrinogen receptors (Fig 4). Despite this, there was no reduction in the percentage of platelets binding PAC1. This suggests that with sustained platelet activation, fibrinogen had become irreversibly bound to a fraction of the fibrinogen receptors on each platelet (Fig 4).

Detection of Activated Platelets in Whole Blood With Monoclonal Antibodies

Flow cytometric analysis of whole blood using the one-color method. Having demonstrated the feasibility of using PAC1 to detect activated platelets after gel filtration, we asked whether similar studies could be carried out in whole blood. To be successful, whole blood analysis would have to include all platelets but exclude all erythrocytes and WBCs. Two different methods were evaluated to accomplish this. In the one-color method, platelets were identified on the basis of their light-scatter profile (Fig 5A). The forward- and side-scatter profiles of platelets could be clearly separated from the larger erythrocytes and WBCs, and a gate could be set around the platelets for analysis. The light-scatter profile of platelets was not affected by the presence of platelet agonists. Studies with gel-filtered platelets and washed erythrocytes confirmed their separation by this method. Approximately 0.5% of gel-filtered platelets appeared outside the platelet

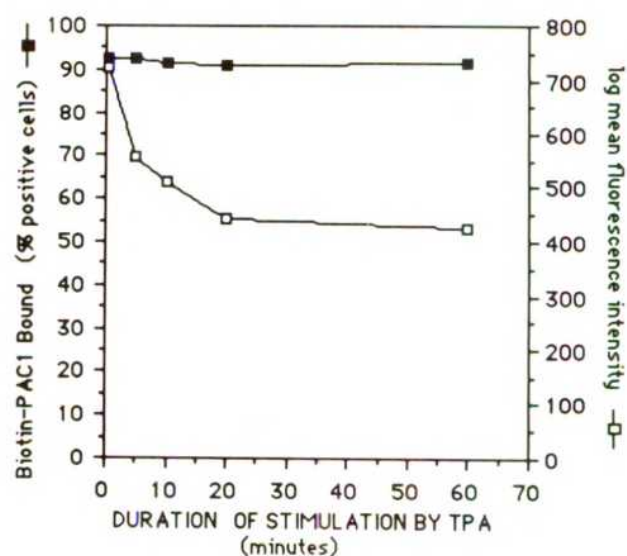


Fig 4. Effect of duration of platelet activation on subsequent binding of biotin-PAC1 to platelets in plasma. Platelet-rich plasma was incubated with 0.2 $\mu\text{mol/L}$ of phorbol myristate acetate (TPA). At the indicated times, biotin-PAC1 was added to the sample, and PAC1 binding was determined by one-color flow cytometry. The percentage of PAC1-positive cells was not affected by the prolonged incubation of activated platelets with fibrinogen, whereas the amount of PAC1 bound per cell (mean fluorescence intensity) did decrease with time.

gate, whereas no more than 0.3% of erythrocytes appeared inside this gate. Thus, assuming a ratio of 20:1 of erythrocytes to platelets in whole blood, at most only 6% of the particles in the platelet gate could have been accounted for by erythrocytes. This agrees well with the observation that ~95% of the cells from whole blood appearing within the platelet gate bound biotin-AP1 or biotin-A2A9, antibodies specific for platelet membrane glycoproteins Ib and IIb-IIIa, respectively.^{27,28} In the experiment shown in Fig 5B, platelets in whole blood were stimulated with ADP and epinephrine in the presence of biotin-PAC1. Following the addition of FITC-streptavidin, the fluorescence of these platelets was compared with that of platelets incubated with PGI_2 to prevent activation. Resting platelets demonstrated minimal fluorescence, similar to control platelets that had not been incubated with antibody. In contrast, platelets stimulated with ADP and epinephrine demonstrated a marked increase in fluorescence.

Flow cytometric analysis of whole blood using the two-color method. Although significant overlap of the platelets with erythrocytes was not a problem in the one-color studies, such overlap may conceivably exist when a different flow cytometer is used or if blood cell light-scatter profiles are abnormal in disease states. Therefore, we evaluated a different approach in which a fluorescent platelet-specific marker, FITC-AP1, was used to identify the platelets in whole blood. Whole blood was incubated first with biotin-PAC1 and then with PE-streptavidin and FITC-AP1. Having bound FITC-AP1, the platelets now exhibited a strong green fluorescence. The machine threshold was then set to exclude from analysis cells that did not exhibit this degree of

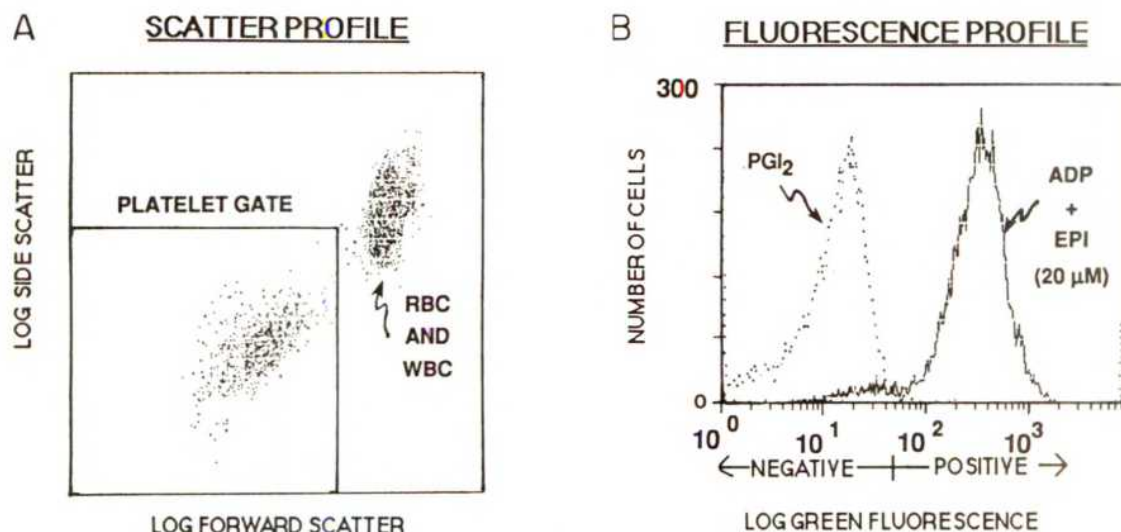


Fig 5. Example of the one-color method of flow cytometric analysis of activated platelets. As described in the Materials and Methods Section, 5 μ L of whole blood were incubated with biotin-PAC1 and with either 1 μ mol/L of PGI₂ to maintain the platelets in a resting state or with 20 μ mol/L of ADP plus epinephrine to expose fibrinogen receptors. Then FITC-streptavidin was added, and the sample was analyzed by flow cytometry. The light scatter data are depicted over 4 log decades (A). The platelets appeared to be separated from erythrocytes and WBCs on the basis of their forward- and side-light scatter, and a gate was placed around the platelets for analysis. For each sample, the cytometer analyzed 10,000 cells within the platelet gate; a histogram of FITC ("green") fluorescence of these cells is shown (B).

green fluorescence (erythrocytes and WBCs), and the platelets were analyzed for biotin-PAC1 binding with PE-streptavidin. Preliminary studies showed that the binding of PAC1 or S12 to platelets did not affect the binding of AP1, nor did AP1 interfere with the binding of the other antibodies. The light-scatter profile for AP1-positive platelets in whole blood showed that ~95% of the cells were in an area expected for single platelets, whereas 5% of the cells demonstrated increased forward- and side-light scatter (Fig 6A).

Separate studies using isolated WBCs and leukocyte-specific antibodies demonstrated that this latter area also contained granulocytes and monocytes (not shown). Thus, since AP1 does not bind to WBCs, this minor population of AP1-positive cells probably represents platelets associated with WBCs. It is unlikely that this population represents platelet aggregates since we found no increase in the percentage of platelets in this scatter area when either platelets in whole blood or gel-filtered platelets were stimulated with agonists.

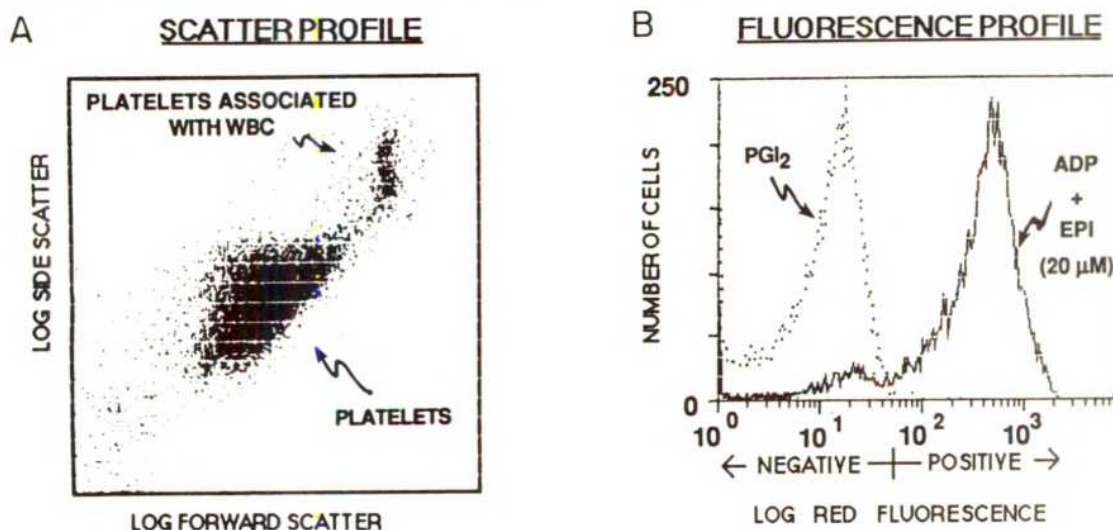


Fig 6. Example of the two-color method of flow cytometric analysis of activated platelets. Whole blood was incubated with biotin-PAC1 and either PGI₂ or ADP plus epinephrine. Then phycoerythrin-streptavidin and FITC-AP1 were added, and the samples were analyzed by flow cytometry. Light-scatter profile of AP1-positive cells (A). These platelets exhibited two separate light-scatter populations. About 95% of the cells appeared to be single platelets, whereas 5% of the platelets exhibited greater light scatter. Once the platelets were identified by their AP1 binding, biotin-PAC1 binding was quantitated by analyzing the platelets for phycoerythrin ("red") fluorescence (B).

When the AP1-positive cells were analyzed for biotin-PAC1 binding, activated platelets showed increased PE fluorescence (Fig 6B). There was no difference in the degree of PAC1 binding between platelets in the major and minor light-scatter profiles. On the other hand, platelet stimulation with epinephrine, ADP, or phorbol ester did not cause significant changes in AP1 binding. In the present studies, the one-color and two-color methods of platelet analysis yielded similar results. Therefore, in the experiments presented below, these methods were used interchangeably.

Effect of platelet agonists on PAC1 and S12 binding. When whole blood was incubated with platelet agonists, the binding of biotin-PAC1 was dependent on the concentration of the agonist (Fig 7). Dose-response curves for epinephrine, ADP, and TPA in whole blood were essentially identical to the corresponding dose-response curves for platelets in plasma or buffer. Moreover, PAC1 binding in response to a submaximal concentration of ADP was augmented by the addition of a low concentration of epinephrine (Fig 7).

S12 is an antibody that recognizes a 140-kd α -granule membrane protein which fuses with the platelet plasma membrane during secretion.^{13,18} The binding of saturating concentrations of biotin-S12 was compared with the binding of biotin-PAC1 to platelets in whole blood. Platelets activated with ADP or ADP plus epinephrine demonstrated binding of both antibodies. At a given concentration of agonist, however, there was more PAC1 than S12 binding (Fig 8). This was expected because unstirred platelets stimulated with weak agonists undergo minimal secretion yet are capable of expressing most of their fibrinogen receptors.⁹ In contrast, platelets stimulated maximally with TPA bound maximal amounts of PAC1 and S12 (Fig 8).

Stability of platelets in whole blood. The above studies were carried out using whole blood that had been processed

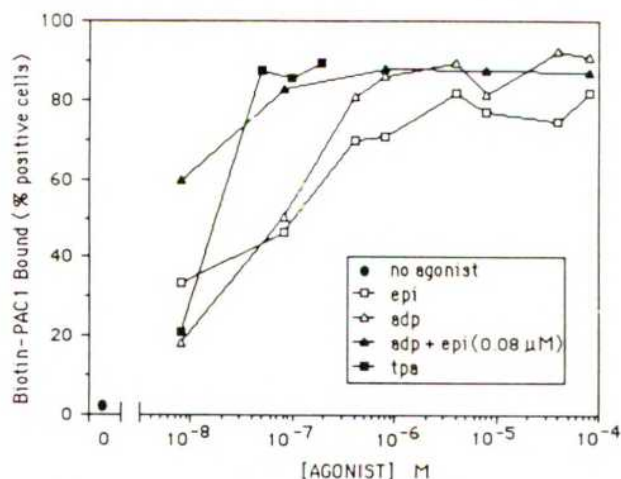


Fig 7. Effect of agonists on the binding of biotin-PAC1 to platelets in whole blood. Whole blood was incubated with the indicated concentrations of agonists and biotin-PAC1. Then PE-streptavidin and FITC-AP1 were added, and the platelets were analyzed for PAC1 binding by the two-color flow cytometry method. The experiment is representative of three so performed. TPA refers to phorbol myristate acetate.

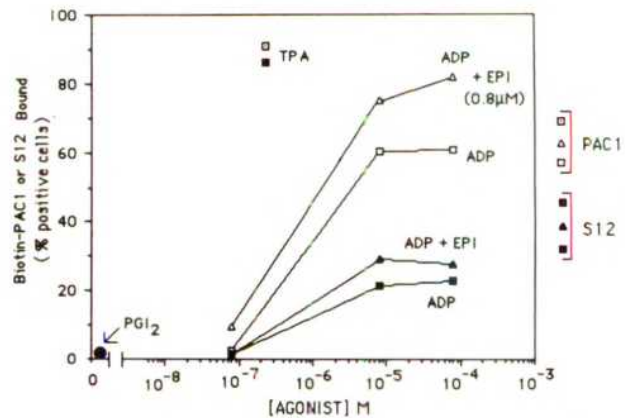


Fig 8. Comparison of the binding of the monoclonal antibodies, PAC1 and S12, to platelets in whole blood. Whole blood was incubated with various agonists and either biotin-PAC1 (open symbols) or biotin-S12 (closed symbols). The samples were then analyzed by one-color flow cytometry, and the amount of antibody bound to the platelets was determined. The experiment is representative of three so performed.

within 1 minute of venipuncture. If the testing of clinical samples is to become feasible, however, it is essential that platelets not become activated spontaneously before analysis. Therefore, we examined the stability of both resting and stimulated platelets during longer periods of incubation. Whole blood containing 0.38% sodium citrate was placed in a capped polypropylene tube. At various times up to 60 minutes aliquots of the blood were incubated with biotin-PAC1 or biotin S12 and then evaluated by flow cytometry. If the blood was allowed to stand for >10 minutes before the addition of antibody, there was a small but perceptible increase in the percentage of platelets binding either PAC1 or S12 (Fig 9). Once either antibody was added to resting or stimulated platelets, however, the platelets maintained their initial degree of platelet activation for up to 2 hours (not shown). Thus, if unfixed blood is to be analyzed and spontaneous platelet activation avoided, the blood specimen should be processed within 10 minutes and analyzed within 2 hours of collection.

It may be necessary to study blood samples from patients hours or days after blood collection. Therefore, the effect of fixing whole blood with 1% paraformaldehyde was examined. Whole blood was fixed as described by George and colleagues,¹⁹ either within seconds of venipuncture or 15 minutes after activation with phorbol ester. Then biotin-PAC1 or S12 was added. Although fixation of blood before the addition of the antibodies was successful in preventing the small time-dependent spontaneous activation of platelets that had been observed with unstimulated and fixed platelets, this procedure resulted in a significant reduction in PAC1 and S12 binding to stimulated cells (Fig 10). In contrast, when the blood was sequentially stimulated with an agonist, incubated with the antibodies and then fixed, PAC1 and S12 binding was similar to that observed for binding to stimulated, unfixed platelets (Fig 10). Furthermore, the amount of antibody bound to platelets fixed after antibody addition was stable for at least 24 hours. Similar results with

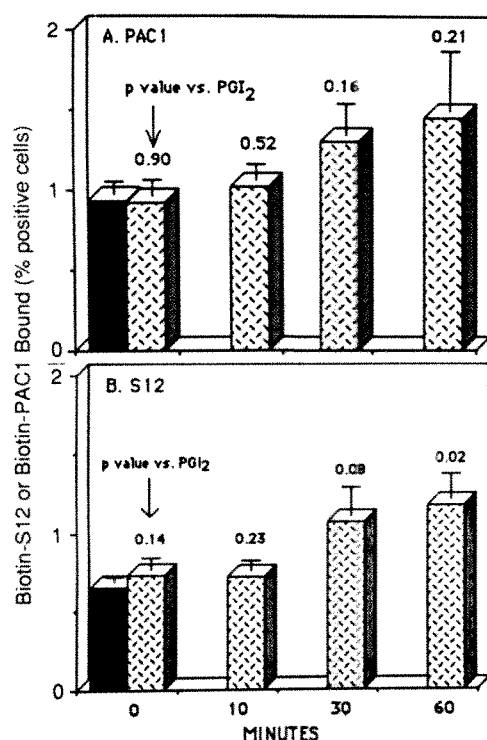


Fig 9. Effect of incubation time on the spontaneous activation of platelets in whole blood. Whole blood anticoagulated with 0.38% sodium citrate was either processed immediately for flow cytometry or allowed to stand in a capped polypropylene tube at room temperature for periods of 0 up to 60 minutes. At the indicated times, aliquots of blood were incubated with saturating concentrations of biotin-PAC1 (A) or biotin-S12 (B), and antibody binding was determined by two-color flow cytometry. The scale on the ordinates range from 0% to only 2% positive platelets. The *P* values compare the data at each time point with that obtained at zero time on platelets incubated with PGI₂ (solid bars). The data represent the mean $\pm$ SEM of platelets from ten normal donors.

fixed platelets were obtained when the antibodies were conjugated with FITC instead of biotin.

DISCUSSION

To function during hemostasis, platelets must become activated. Platelets may also become activated in certain pathologic conditions and participate in the process of thrombogenesis.^{21,22} In the present studies, we sought to develop a direct method to detect activated platelets in whole blood specimens immediately after venipuncture and without the need to centrifuge or wash the specimen. We took advantage of the availability of PAC1 and S12, two monoclonal antibodies that bind specifically to activated but not to resting platelets. PAC1 detects a change in the glycoprotein IIb-IIIa complex occurring during platelet activation that converts the complex into a competent fibrinogen receptor.⁹ S12 detects an α -granule membrane protein that becomes exposed on the platelet surface coincident with granule secretion.^{13,18} The binding of these antibodies to activated platelets was detected by flow cytometry. The antibodies were conjugated with biotin, and binding was detected with streptavidin conjugated either with fluorescein or PE. Plate-

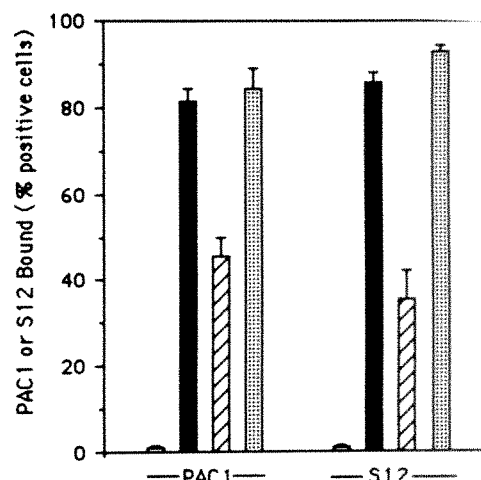


Fig 10. Effect of fixation with 1% paraformaldehyde on PAC1 and S12 binding to platelets in whole blood. Whole blood was incubated for 15 minutes with either biotin-PAC1 or biotin-S12; then FITC-streptavidin was added, and the amount of antibody bound to the platelets was determined by one-color flow cytometry. Open bars represent platelets from unfixed blood incubated with PGI₂ during the antibody and FITC-streptavidin steps. Closed bars represent platelets from unfixed blood stimulated with 0.2 μ mol/L of phorbol myristate acetate for 15 minutes during the incubation with antibody. Striped bars represent platelets from blood stimulated with the phorbol ester for 15 minutes and then fixed with paraformaldehyde before the antibody incubation steps. Stippled bars are platelets from blood that was stimulated with 0.2 μ mol/L of phorbol myristate acetate and fixed after the incubations with antibody and FITC-streptavidin. Data represent the mean $\pm$ SEM of seven experiments.

lets activated with agonists *in vitro* bound the fluorescently tagged antibodies and were distinguishable from resting platelets on the basis of their fluorescence intensity. The amount of platelet-bound antibody measured by flow cytometry correlated strongly with the amount of bound ¹²⁵I-antibody measured in a standard filtration assay. Antibody binding to platelets in whole blood was dependent on the agonist concentration, and the dose-response curves were similar to those for platelets in plasma or buffer. Furthermore, mixing studies showed that if as little as 0.8% activated platelets was present in the platelet sample, they could be detected by flow cytometry. Thus, the method described appears to be a sensitive and direct one for the detection of activated platelets.

Precise analysis of platelets in whole blood requires that these cells be clearly differentiated from erythrocytes and WBCs by the flow cytometer. Two different protocols were established for this purpose. In the one-color method, platelets were identified by their light-scatter profile. Under the conditions used, there was minimal overlap between platelets and the larger blood cells so that only ~5% of the cells in the platelet scatter area could be accounted for by erythrocytes. Therefore, for platelet analysis, a gate was set around this scatter area, and antibody binding was determined by quantitating platelet fluorescence. When platelets were stimulated by an agonist but not stirred, the activated platelets exhibited the same forward- and side-scatter profiles as

resting platelets. Although the one-color method was acceptable for the present studies, it has potential disadvantages. First, it may not be possible to so clearly differentiate platelets from erythrocytes on the basis of light scatter using other flow cytometers.²⁵ Second, blood in this study was obtained from normal volunteers. Platelets may show more light scatter overlap with erythrocytes in certain disease states. Third, we found that ~5% of the platelets in whole blood demonstrated a light-scatter profile similar to that of WBCs, consistent with the report of Jennings and colleagues that platelets can be loosely associated with granulocytes or monocytes.²⁵ This minor population of platelets would not be analyzed by the one-color method. Although the percentage of platelets apparently associated with WBCs did not change with platelet activation, this may not be the case in disease states. To overcome these potential drawbacks, we evaluated a different two-color method of analysis that did not depend on light scatter to identify platelets.

In the two-color method, platelets were labeled with biotin-PAC1 or S12 and then identified with the platelet-specific antibody, FITC-API, which recognizes platelet membrane glycoprotein Ib. The cytometer threshold was set to analyze only FITC-API-positive cells, thereby eliminating erythrocytes and WBCs from the analysis. Biotin-PAC1 or S12 binding was then detected with PE-streptavidin, and activated platelets were identified by their PE fluorescence. When using this method to differentiate platelets from other blood cells, it is important to use an antibody to detect platelets whose binding does not change significantly with platelet activation. Although API binding decreases during thrombin-induced platelet activation,^{29,30} we did not observe sufficient changes in API binding with ADP, epinephrine, or phorbol ester to preclude its use as a platelet marker for two-color analysis.

Several variations of this two-color method could increase its range of usefulness for specific applications. For example, the activation-dependent antibodies can be directly labeled with FITC, and the API could be labeled with biotin. Indeed, S12 has been simultaneously labeled with FITC and ¹²⁵I to correlate directly the antibody binding observed in flow cytometric and radioisotopic assays.²⁶ It should also be possible to label PAC1 with FITC and S12 with biotin and evaluate the binding of these two activation-dependent antibodies simultaneously.

Whole blood flow cytometry for the study of activated platelets has several advantages. First, only microliter volumes of blood are required, making it possible to study infants or individuals with thrombocytopenia. Second, as illustrated in Fig 5, platelet subpopulations that are heterogeneous in their platelet response to stimulation can be detected, which is not possible with methods that rely on ¹²⁵I-antibody binding or on measurements of released platelet factor 4 or β -thromboglobulin. Third, the use of whole blood eliminates the need for centrifugation or washing. These procedures can result in platelet activation. No method of *ex vivo* analysis, however, will prevent activation of platelets that may take place during a traumatic or prolonged venipuncture. Thus, close attention must be paid to the details of venipuncture. In this study, we attempted to minimize acti-

vation by drawing blood from the antecubital vein with the subject supine, using little or no tourniquet, and discarding the initial 2 mL of blood.

The stability of platelets *in vitro* is a major variable in these types of studies. We found that spontaneous activation of platelets did not occur if PAC1 or S12 was added to the blood sample within 10 minutes of venipuncture. A small but detectable increase in PAC1 or S12 binding was observed, however, if blood was allowed to stand for longer periods of time before the addition of antibody. Therefore, if a direct assay for activated platelets is to gain wider use, it may be desirable to fix the blood sample after venipuncture. Fixation of the blood with 1% paraformaldehyde immediately after venipuncture prevented spontaneous platelet activation. If platelets were stimulated with an agonist and then fixed, however, antibody binding to the fixed platelets was significantly reduced as compared with unfixed platelets. This problem was circumvented by adding the paraformaldehyde *after* the antibody. The effects of platelet fixation for each antibody used must be carefully evaluated.

Whether the direct analysis of platelets will be useful in detecting and quantitating the degree of platelet activation in clinical disorders remains to be established. Based on studies with plasma platelet factor 4, β -thromboglobulin, and the plasma and urinary metabolites of thromboxane A₂, it appears that platelet activation can accompany unstable angina, adult respiratory distress syndrome, cardiopulmonary bypass, and renal dialysis.^{21,22,31,32} In addition, it is conceivable that activated platelets circulate in other disorders, such as thrombotic thrombocytopenic purpura. Even if a fraction of the platelets activated in these conditions continued to circulate in the blood, they might be detectable using activation-dependent monoclonal antibodies. Indeed, George and co-workers, using ¹²⁵I-S12, found activated platelets in 46% of patients with adult respiratory distress syndrome but in only 11% of patients undergoing cardiopulmonary bypass.¹⁹ Although this may indicate that the mechanism of platelet activation varies in different clinical disorders, the results may also be accounted for by the detection limits of the assay. The present studies suggest that assay sensitivity may be increased by using flow cytometry rather than conventional radioligand binding methods and by using a panel of activation-dependent monoclonal antibodies. Nonetheless, careful studies will be required to establish the sensitivity and specificity of the flow cytometry technique and its clinical value in detecting activated platelets in humans.

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CONCISE REPORT

Physiologic Regulation and Tissue Localization of Renal Erythropoietin Messenger RNA

By Stephen J. Schuster, Jean H. Wilson, Allan J. Erslev, and Jaime Caro

Although erythropoietin (Epo) is produced primarily by the kidneys in response to hypoxia, the precise cell type(s) and mechanisms by which these cells regulate production are poorly understood. In the experiments we report, the kinetics of renal Epo production in response to acute hypoxia and the intrarenal localization of cellular Epo synthesis were studied at the level of Epo mRNA. Erythropoietin mRNA expression was determined by Northern blot analysis of rat kidney RNAs using a probe derived from the mouse Epo gene. Renal Epo mRNA content increased as early as 1 hour after initiation of hypoxia and continued to accumulate during 4 hours of stimulation. Discontinuation of the hypoxic stimulus resulted in rapid decay of mRNA

levels. Kidney and plasma Epo levels measured by radioimmunoassay paralleled, with respective lag times, the changes in renal Epo mRNA content, suggesting that Epo production in response to acute hypoxia represents de novo synthesis and is regulated by changes in Epo mRNA. Northern blot analysis of RNAs extracted from separated glomerular and tubular tissue fractions revealed Epo mRNA in the tubular fraction, whereas glomerular tissue did not contain Epo mRNA. Thus, the site of cellular Epo synthesis is located in the renal tubule or its interstitium and not in the glomerular tuft.

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THE GLYCOPROTEIN growth factor erythropoietin (Epo) is produced primarily by the kidneys¹ and stimulates proliferation and differentiation of erythroid progenitor cells in the bone marrow.² The plasma Epo level increases in response to hypoxia and thus regulates production of mature, oxygen carrying RBCs to meet tissue requirements. Recently, Epo mRNA was shown to accumulate within the kidney in response to acute anemia³ and cobalt exposure.⁴ However, the kinetics of Epo mRNA changes in response to hypoxic hypoxemia and the precise cellular localization of Epo production within the kidney remain unknown. A glomerular origin of renal Epo has been suggested by immunofluorescence studies⁵ and by reports of Epo production by in vitro glomerular⁶ and mesangial cell⁷ cultures. In contrast, the greater intracellular Epo content in tubular tissue⁸ and in vitro Epo production by a proximal tubular cell line⁹ support a tubular cell site of renal Epo synthesis. In this report, we describe the kinetics of renal Epo mRNA expression in response to acute hypoxia and relate this to renal Epo production. Furthermore, we localize Epo mRNA within the kidney to the nonglomerular tissue.

MATERIALS AND METHODS

For the kinetic studies, adult male Wistar rats were placed in a hypobaric chamber at 0.4 atmosphere, and kidneys were obtained

from groups of animals removed at 1, 2, and 4 hours after the onset of hypoxia. After 4 hours of hypobaric hypoxia, kidneys were obtained from groups of rats kept at normobaric conditions for 1, 2, and 3 hours posthypoxia. PolyA⁺ RNA was prepared from whole kidney homogenates in each experimental group and Northern blot analysis was performed as described below. Epo content of kidney extracts and plasma for each experimental group was determined by radioimmunoassay as previously described.⁸

Localization of Epo mRNA within specific kidney tissue fractions was studied in animals whose Epo production had been maximally stimulated by combining exposure to hypoxia and cobalt. Kidneys were obtained from animals injected with cobalt chloride (6 mg/100 g of weight) and exposed 12 hours later to 4 hours of hypobaric hypoxia at 0.4 atmosphere. The cortical tissue was separated into glomerular and tubular fractions using a differential sieving technique as previously described.⁸ The first fraction consisted of a mixture of intact and decapsulated glomeruli as verified by phase and electron microscopy studies. The second fraction consisted of intact tubular fragments and single tubular cells with a small amount of interstitial cell contamination. The tubular origin of this fraction was confirmed by histologic studies and by measurement of specific brush border membrane enzymes.¹⁰ PolyA⁺ RNA was extracted from both fractions as well as from stimulated and control whole kidneys, and Northern blot analysis was performed.

Total RNA was extracted from whole kidney homogenates and kidney tissue fractions using the guanidinium-cesium chloride technique,¹¹ and polyA⁺ RNA was selected using oligo(dT)-cellulose chromatography.¹² PolyA⁺ RNA samples (10 µg) were denatured in formamide-formaldehyde and electrophoresed in a 1.4% formaldehyde/agarose gel as described.¹² RNAs were then transferred to a Nytran membrane (Schleicher and Schuell) in 10× standard saline citrate (SSC) and hybridized with a complementary strand RNA probe for Epo mRNA. The Epo probe was derived from a 660-base pair (bp), *SmaI-EcoRI* fragment of the mouse Epo gene inserted into a pSP65 plasmid. This genomic fragment represents 160 bases of intron IV and 500 bases of exon V.^{13,14} Complementary strand RNA probes were generated by in vitro transcription by SP6 RNA polymerase of the coding strand of the insert, using ³²P-cytidine triphosphate as previously described,¹⁵ and yielded RNA probes with specific activity of 10⁸ cpm/µg. Northern blots were hybridized at 65°C with probe (10⁷ cpm/mL) in 50% formamide, 10% dextran sulfate (wt/vol), 20 mmol/L of NaPO₄, pH 6.5, 5× SSC, 0.2% sodium dodecyl sulfate (SDS), 0.05% bovine serum albumin (BSA), 0.05% Ficoll, 0.05% polyvinylpyrrolidone (PVP), 200 µg/mL of denatured salmon sperm DNA, 1 mg/mL yeast tRNA, and 100

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$\mu\text{g/mL}$ each of poly A, poly I, and poly C. After hybridization for 12 hours, nylon filters were washed under progressively more stringent conditions with final wash in $0.1 \times \text{SSC}$, 0.1% SDS at 65°C . Washed filters were further digested with RNase A¹⁶ and exposed for 12 to 24 hours to Kodak XAR-5 film at -70°C using intensifying screens.

Control Northern blots were hybridized with a mouse glyceraldehyde-3-phosphate dehydrogenase cDNA probe labeled by nick-translation to a specific activity 10^8 cpm/ μg of DNA using ^{32}P -deoxycytidine triphosphate.¹⁷ Hybridization conditions were similar to those described above except that hybridization and stringency washing were at 42°C . Northern blots hybridized with nick-translated probe were not digested with RNase A following stringency washes.

RESULTS

To study the kinetics of Epo mRNA expression, polyA⁺ RNA was extracted from whole rat kidney homogenates at various times during a 4-hour hypoxic challenge and at various intervals posthypoxia. The analysis of Northern blots (Fig 1) revealed Epo mRNA as early as 1 hour after initiation of hypoxia with progressive accumulation during 4 hours of observation. During the posthypoxic state, there was a sharp decline in mRNA with 50% of the signal lost in the first hour and none detectable 3 hours after discontinuation of the stimulus. The observed signal for Epo mRNA originated at 1.8 kilobases (kb), which is consistent with the size previously reported by Beru and colleagues.⁴

To relate Epo mRNA levels with changes in protein production, measurements of the change in the Epo content of the kidney and plasma were made in similarly treated groups of animals using a sensitive Epo radioimmunoassay.⁸ As shown in Fig 2, the intrarenal level of Epo starts to change after 1 hour of hypoxia, reaching its peak after 4 hours of stimulation. During the posthypoxic period, there is a rapid decline in renal Epo. The plasma levels of the hormone

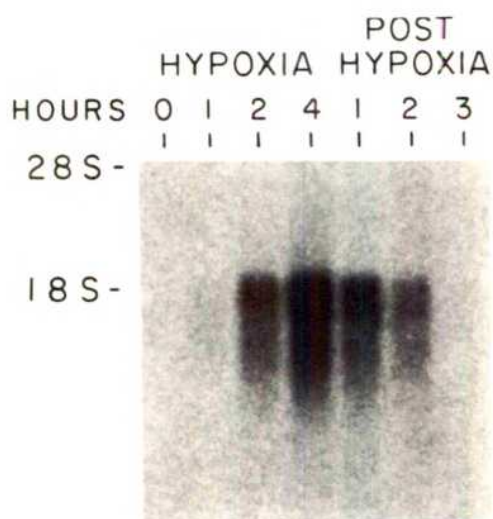


Fig 1. Northern blot analysis of RNA from rat kidneys obtained at various times after the onset of hypoxia and during the posthypoxic period. PolyA⁺ RNAs were prepared for each time point and hybridized with a complementary strand RNA probe for erythropoietin (Epo) mRNA.

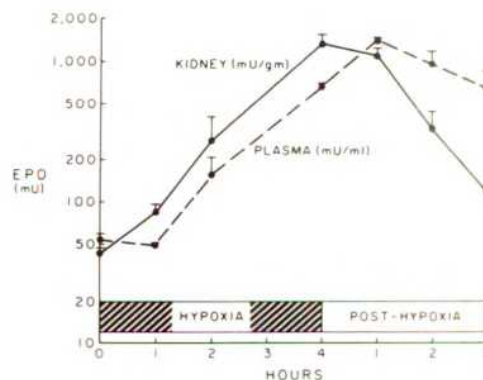


Fig 2. Changes in plasma and kidney erythropoietin (Epo) content in rats at various times after onset of hypoxia and during the posthypoxic period. Experimental design was the same as in Fig 1. Epo levels in plasma and kidney homogenates were measured by a sensitive radioimmunoassay as previously described.⁸ Results are expressed as the mean $\pm$ SE of two or three independent measurements.

parallel, with a short lag time, the changes seen in kidney Epo content and Epo mRNA.

To localize cellular Epo synthesis within the kidney, kidneys from stimulated rats were separated into glomerular and tubular tissue components, and tissue fractions were probed independently for Epo mRNA content. Northern blot analysis (Fig 3A) showed an intense signal in stimulated whole kidney and in the corresponding tubular fraction RNA, whereas no signal was detected in glomerular fraction RNA. The difference in intensity of signal between total kidney and tubular fraction RNAs can be explained in part

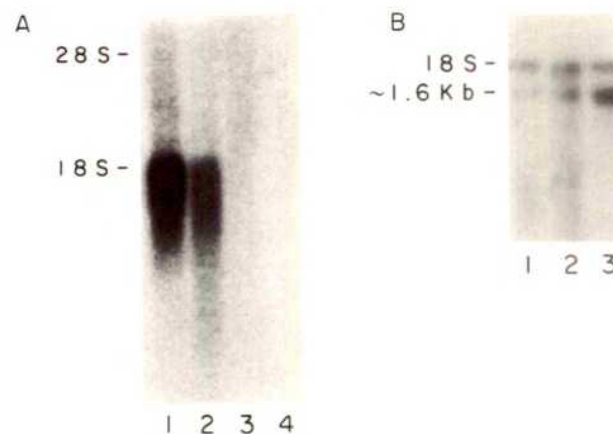


Fig 3. Localization of erythropoietin (Epo) mRNA in whole kidneys and separated kidney tissue fractions. Kidneys were obtained from hypoxic animals and separated by a sieving technique into the glomerular and tubular fractions.⁸ PolyA⁺ RNA was extracted, and northern blot analysis was performed as described. Hybridization with ^{32}P -labeled Epo RNA probe (A). Lane 1, stimulated whole kidney ($10 \mu\text{g}$); lane 2, stimulated tubular fraction ($20 \mu\text{g}$); lane 3, stimulated glomerular fraction ($20 \mu\text{g}$); and lane 4, normal whole kidney ($10 \mu\text{g}$). Hybridization with ^{32}P -labeled nick-translated¹⁷ glyceraldehyde-3-phosphate dehydrogenase cDNA probe (B). Lane 1, stimulated whole kidney ($10 \mu\text{g}$); lane 2, stimulated tubular fraction ($10 \mu\text{g}$); and lane 3, stimulated glomeruli ($10 \mu\text{g}$).

by the short half-life of the mRNA, since the separation procedure takes ~30 minutes. Quantitation of mRNAs in the different fractions was verified by hybridization with a ³²P-labeled glyceraldehyde-3-phosphate dehydrogenase cDNA probe, as shown in Fig 3B.

In whole kidney and tubular fraction RNAs, there appeared to be significantly more degradation of the Epo mRNA as compared with glyceraldehyde-3-phosphate dehydrogenase message. Technical factors may have contributed to this appearance, however, since the autoradiographs probed for Epo mRNA were intentionally overexposed to enhance subtle differences in expression. In addition, the single-stranded RNA probe for Epo mRNA is more sensitive than the nick-translated glyceraldehyde-3-phosphate dehydrogenase cDNA probe; thus, small amounts of degraded mRNA are more easily detected.

DISCUSSION

The early studies by Jacobson and co-workers¹ in nephrectomized animals clearly established the kidney as the primary source of Epo in adults. Although the ability of the kidney to produce Epo directly had been questioned,¹⁸ probes derived from the recently cloned human and mouse Epo genes have provided evidence for the direct participation of the kidney in the biogenesis of Epo.^{3,4} Our experiments demonstrate that renal Epo mRNA begins to accumulate within 1 hour of exposure to hypoxia and this accumulation parallels the rise in kidney and plasma Epo content. Discontinuation of hypoxia results in rapid decline in the level of Epo mRNA, followed by a decrease in kidney and plasma Epo. Renal Epo synthesis thus appears to be regulated by the level of its specific mRNA and these levels appear to be highly sensitive to variation in tissue oxygenation. Although

variations in mRNA probably reflect changes at the transcriptional level, possible regulatory effects influencing mRNA stability cannot be ruled out. In addition, our results suggest that renal Epo production in response to hypoxia represents *de novo* synthesis with rapid release and no significant intracellular Epo accumulation.

The localization of Epo production within the kidney has been a source of contention. Immunofluorescence studies have localized Epo to the glomeruli of hypoxic kidneys and suggested a glomerular origin of renal Epo.⁵ Furthermore, Epo has been reported to exist in extracts from isolated glomeruli of rats exposed to hypobaric hypoxia¹⁹ and that Epo-like activity can be demonstrated in conditioned medium from *in vitro* cultures of glomerular-mesangial cells.⁷ On the other hand, a tubular origin of renal Epo was suggested by Caro and Erslev,⁸ who demonstrated that the major portion of intracellular Epo in the kidney is contained in tubular cell extracts. In addition, these authors demonstrated Epo production by a proximal tubular cell line.⁹ Our studies have localized Epo mRNA to the tubular cell fraction of sieve-separated kidney tissues with no detectable Epo mRNA in the glomerular fraction. The renal tubular fraction is histologically complex, with tubular segments, interstitium and vascular elements. A precise localization of Epo mRNA within this fraction will require *in situ* hybridization experiments.

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CONCISE REPORT

The Effect of the Plasticizer Di(2-ethylhexyl)phthalate on Red Cell Deformability

By R.S. Labow, R.T. Card, and G. Rock

Red cell concentrates (RCC) are stored for 35 to 42 days in plastic containers manufactured with the liquid plasticizer di(2-ethylhexyl)phthalate (DEHP). DEHP leaches from the polyvinylchloride (PVC) plastic bag, then binds to and stabilizes the RC membrane. This study was undertaken to determine the deformability of the RC membrane using an osmotic gradient ektacytometer and to relate these measurements to the concentration of DEHP in the stored RCC. Pooled RCC was aliquoted into PL146 (PVC), PL732 (polyolefin), and PL732 (with added DEHP) bags with samples removed weekly for analysis of osmotic fragility, deformability, and DEHP concentration. The adenosine triphosphate (ATP) content was also measured. The increase in osmotic fragility during storage was greater when RCC

was stored without DEHP. In addition, there was a decrease in the maximum elongation index (EI max) when there was decreased DEHP in the storage bag. The osmolality (Omax) at which EI max occurred, as well as the Omin, the osmolality at which minimum elongation (EI min) occurred was higher in the PL732 container than in the PL146 or in the PL732 to which DEHP had been added. These changes could be reversed by addition of DEHP at the beginning of the storage period, showing a direct correlation between DEHP concentration during storage and RC membrane flexibility. By a better understanding of the mechanism of DEHP protection, it might be possible to substitute a less toxic stabilizing compound.

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IT IS WIDELY ACCEPTED that impaired red cell (RC) deformability can cause a reduction in cell survival.^{1,2} During storage in the liquid state, RCs progressively lose their viability³; this loss is associated with loss of membrane lipid⁴ and with reduced deformability.⁵ Deformability of RC membranes can be measured using osmotic gradient ektacytometry. Characteristic profiles are obtained and can be used to detect changes in membrane surface area and water content. Clark et al⁶ have shown that by selectively altering the membrane area and water content of normal cells three features of the osmotic deformability profile can be used to characterize cell populations: (1) The osmolality at which hypotonic minimum deformability or elongation (EI min) occurs, designated Omin, provides a measure of the average ratio of surface area to volume of the cell population. This value coincides with that of the osmolality at which 50% of the cells hemolyze in an osmotic fragility test. (2) For homogeneous cell populations with normal or reduced lipid content, the height of the maximum deformability or elongation index (EI max) reflects the amount of membrane surface area available for cell deformation. The position of the EI max moves to lower or higher osmolality (Omax) as the water content of the cell is decreased or increased, whereas a reduction in membrane surface area causes a reduction in maximum attainable EI. (3) A measure of initial water content of the cell can be derived from the hypertonic area of the profile by determining the osmolality at which EI reaches a particular value, since cell deformation is also determined by intracellular viscosity.

Polyvinylchloride (PVC) plastic is the most commonly used material in medical devices and was the first plastic to be used for the storage of whole blood.⁷ The plasticizer di(2-ethylhexyl)phthalate (DEHP) that is added to PVC plastic to impart flexibility, leaches from the plastic into plasma as was first reported in 1967 by Guess et al.⁸ Since that time DEHP has been found in any situation where lipid-containing biological fluids come into contact with DEHP-containing PVC plastic.⁹

Chronic toxic effects such as carcinogenicity,¹⁰ embryotoxicity,¹¹ and testicular atrophy¹² have been attributed to

DEHP and its major metabolite mono(2-ethylhexyl)phthalate (MEHP) in rodents. In order to provide blood component therapy free of these plasticizer contaminants, it would be valuable to search for non-DEHP plastic material that could be used for component storage. Recently, non-DEHP plastic bags have been introduced for platelet storage because of their enhanced gas permeability. The most commonly used materials are polyolefin (PO) plastic (with no plasticizer in the formulation) or PVC plastic with tri(2-ethylhexyl)trimellitate (TOTM) instead of DEHP as the plasticizer. Although the non-DEHP bags have increased the storage time of platelets from three to five days¹³ due to the better gas exchange, it was found that RCs are only stable for 21 days when stored in non-DEHP plastic.¹⁴ We¹⁵ and others^{16,17} have found that the DEHP that leaches out of the PVC bags binds to the RC membrane and stabilizes it. After day 14 the osmotic fragility and plasma-free hemoglobin increases much more rapidly when the RCs are stored in non-DEHP plastic^{14,17} than if stored in PVC-DEHP bags. Osmotic fragility and plasma-free hemoglobin have been correlated

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directly with DEHP concentration during storage.^{15,16} In order to try to elucidate the mechanism of the DEHP protection, we measured the RC deformability using the osmotic gradient ektacytometer and correlated this to the DEHP concentration during storage and have related the characteristic measurements (Omin, EI max, Omax) to the osmotic fragility and adenosine triphosphate (ATP) content measured on the same samples.

MATERIALS AND METHODS

Preparation of RC concentrate. Whole blood was collected from O-positive donors who met all the criteria of the Canadian Red Cross Blood Transfusion Service. RC concentrate (RCC) was prepared using standard blood bank procedures. RCC (from two donors) was pooled and aliquoted by weight into three containers; the PL146 bag (PVC standard RCC storage container that is routinely used), the PL732 (polyolefin container normally used for platelet concentrate storage) to which dimethylsulfoxide (DMSO) was added, and the PL732 container with added DEHP dissolved in the same amount of DMSO. There was also a PL732 container used with no additions, and a PL146 container with DMSO added as additional controls. The DEHP was dissolved in DMSO to give a final concentration of 150 $\mu\text{g/mL}$ RCC. A stock solution of DEHP in DMSO (100 mg/mL) was prepared. DEHP 30 to 40 mg was added to give a final concentration in each unit of RCC of 150 $\mu\text{g/mL}$. The amount added was adjusted to the exact volume of RCC in the container. These three containers were stored under standard conditions. On days 0, 7, 14, 21, 28, and 35 a sample was removed from each container using sterile technique and analyzed for osmotic fragility using the method of Roy and Neteboom.¹⁸ The DEHP concentration was measured by high performance liquid chromatography (HPLC) using a modification of a previously published method.^{19,20} This study was performed in triplicate, ie, three pools of two donors for each pool were aliquoted into nine containers using the three conditions described. Since the determination for each data point was done in duplicate, the number of observations was six. This includes the ektacytometry measurements as well. All plastic containers were obtained from Fenwal Corporation, Deerfield, IL. The ATP concentrations were measured on each sampling day, according to the procedure of Beutler.²¹

Measurement of RC deformability. Part of the sample removed on days 0, 7, 14, 21, 28, and 35 (1.0 mL) was placed in a small plastic tube, packed in ice, and shipped by air at 4°C to Saskatoon, where the deformability measurements were performed the same day using a Technicon ektacytometer (Technicon Instruments Corporation, Tarrytown, NY) in the osmoscan mode. In addition, RCC was prepared and stored under standard conditions at the Saskatoon Center and the results of RC deformability compared with the RC samples that were shipped by air from Ottawa. The method consists of subjecting RC in 3.1% polyvinylpyrrolidone (PVP) to a constant fluid shear stress over an osmolality gradient from 60 to 600 mosm/kg prepared using phosphate-buffered saline. The resulting deformation was then measured and recorded continuously. The EI is a measure of RC deformability and varies with the osmolality of the suspending medium. The characteristic osmotic profile is defined by a number of indices: EI max is the maximum elongation that occurs in iso-osmolar medium (Omax); EI min is the lowest deformability that occurs in hypotonic medium (Omin).

RESULTS

The DEHP concentrations in each of the storage containers changed as was expected. The variation in the DEHP concentration (Table 1) is due to variation in the amount by

Table 1. DEHP Concentration ($\mu\text{g/mL}$ RCC)

Day	PVC	PO Plus DMSO	PO Plus DEHP in DMSO
0	22 $\pm$ 1.1	2.7 $\pm$ 1.4	155 $\pm$ 57
7	54 $\pm$ 19	4.2 $\pm$ 1.4	95 $\pm$ 10
14	89 $\pm$ 34	6.0 $\pm$ 2.1	78 $\pm$ 3
21	116 $\pm$ 54	7.4 $\pm$ 3.2	85 $\pm$ 11
28	141 $\pm$ 77	8.4 $\pm$ 4.0	78 $\pm$ 17
35	174 $\pm$ 88	9.2 $\pm$ 4.5	66 $\pm$ 12

weight of the pooled RCC in the PVC storage container. In the standard PVC container normally used for RCC storage, DEHP continued to be extracted from the plastic increasing to 174 $\pm$ 88 $\mu\text{g/mL}$ by day 35. When DEHP is added to the PO container at 0 time, there is a drop in concentration with time due to the conversion of DEHP to MEHP.²² The PO container, although manufactured without DEHP, does contain small quantities of this ubiquitous plasticizer^{19,23} of which small amounts (<10 $\mu\text{g/mL}$) are extracted during the storage period. There is less variation when the weights are taken into account in adding DEHP in DMSO to the PO container. However, the difference in DEHP concentration in the PVC and PO containers is so great that it is evident that the results obtained when measuring RC deformability and osmotic fragility were directly related to the DEHP concentration measured in the RCC.

The DMSO used to dissolve the DEHP that was added to both PO containers had no effect on any of the measurements performed in this study. All of the measurements performed on the RCC stored in the PVC container, the PO container plus DMSO, and the PO container to which DEHP in DMSO was added were also performed on RCC stored in a PL146 container to which only DMSO was added, as well as

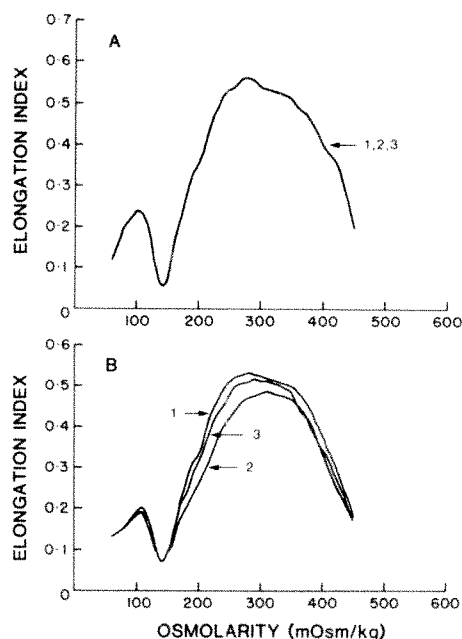


Fig 1. RC deformability measured in RCC stored in three types of bags: PL146, PL732 plus DMSO, and PL732 plus DMSO and DEHP. (A) Day 0; (B) day 35.

in a PL732 container to which nothing was added. This was another check that all observed effects were related to DEHP concentration and not to the DMSO, with the only differences observed being related to the DEHP concentration.

Figure 1 shows the RC deformability for RC storage under all three storage conditions. Using the student's *t* test, there was a statistically significant difference between PL732 plus DMSO alone compared with PL146 or PL732 plus DMSO and DEHP; this can be clearly seen when the EI max, Omax, and Omin values are plotted separately (Fig 2) with error bars. This profile was identical for all three storage conditions on day 0 (Fig 1A). The EI varied with the

osmolality of the suspending medium and EI max occurred in iso-osmolar medium (290 mosm/kg, Omax) as was expected. EI min occurred at 140 mosm/kg (Omin). Figure 1B shows the deformability profiles of the same samples on day 35 of storage. The storage container with no DEHP had a greater loss of deformability EI max (Fig 2A). Omax values (Fig 2B) were shifted to the right, that is, toward higher osmolality. Addition of DEHP to the non-DEHP bag partially corrected the defect. With DEHP present from the start of the storage period there was a reversal Omin shift to higher osmolality (Fig 2C). The Omin values correlated directly with the 50% hemolysis point on the osmotic fragility curves (Fig 3). The data points for the osmotic fragility curves represent the mean of the values obtained from three pools. The standard deviation was calculated for the concentration of sodium chloride at 50% hemolysis for each pool for each sampling day (Table 2). There was no statistically significant difference between the PVC or the PO container in the 50% hemolysis point up to day 21 of storage. After day 21, it was evident that the increased concentration of DEHP caused a decrease in the osmotic fragility. The shift to higher percent sodium chloride was equal to the Omin shift on the deformability profiles on day 35 of storage.

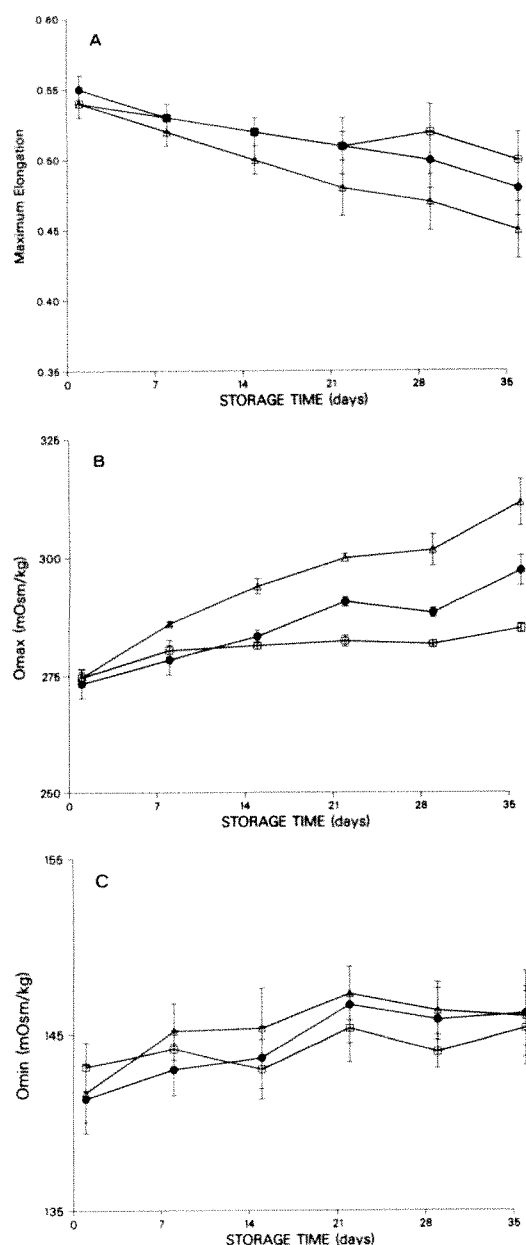


Fig 2. The variation of EI max (A), Omax (B), and Omin (C) with increasing storage time in three types of storage containers: PL146, Δ ; PL732 plus DMSO, $\square$; and PL732 plus DMSO and DEHP, $\bullet$.

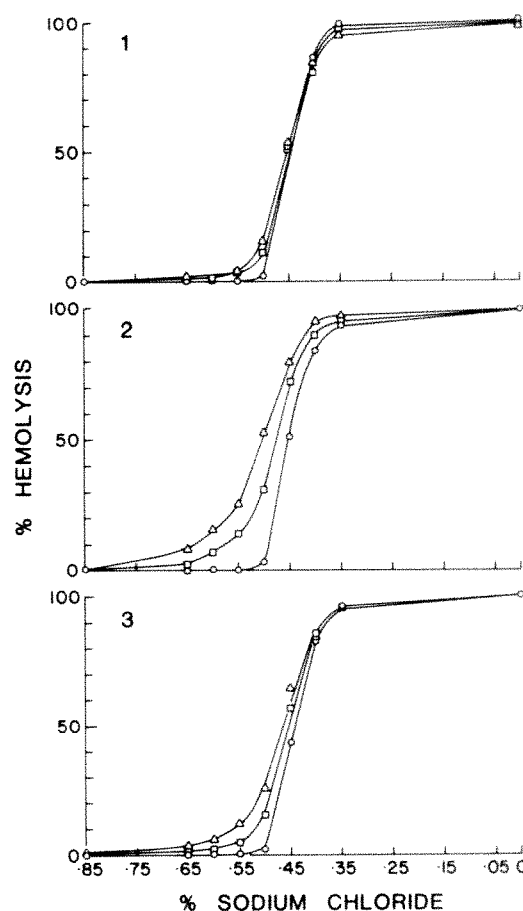


Fig 3. The variation of osmotic fragility with increasing storage time in three types of containers: PL146, PL732 plus DMSO, and PL732 plus DMSO and DEHP. Day 0, $\circ$; day 21, $\square$; and day 35, $\triangle$.

Table 2. Sodium Chloride Concentration at 50% RC Hemolysis

Day	PVC	PC Plus DMSO	PO Plus DEHP in DMSO
0	0.455 ± 0.013	0.448 ± 0.003	0.447 ± 0.008
7	0.452 ± 0.003	0.457 ± 0.008	0.448 ± 0.008
14	0.462 ± 0.016	0.458 ± 0.006	0.460 ± 0.013
21	0.465 ± 0.108	0.487 ± 0.012	0.467 ± 0.016
28	0.462 ± 0.022	0.488 ± 0.013	0.472 ± 0.016
35	0.467 ± 0.017	0.503 ± 0.011	0.482 ± 0.022

The ATP concentration in the RCC decreased with increasing storage time to the same extent in all three types of storage conditions (Fig 4). The differences observed are not statistically significant (student's *t* test).

DISCUSSION

Over the past few years, concern has arisen about the toxic effects of DEHP and its metabolites. We¹⁵ and others^{16,17} have shown that DEHP binds to the RC membrane and lowers its osmotic fragility. When attempts were made to store RCC in non-DEHP containers beyond 21 days, increased osmotic fragility and plasma-free hemoglobin were measured¹⁵ as well as decreased in vivo survival times.¹⁴ Horowitz et al¹⁷ stored RCC in polyethylacrylate bags and showed that RC metabolism was preserved, but the RC membrane had greatly increased fragility. We have studied the changes in RC deformability during storage and have shown that DEHP altered the membrane flexibility. The presence of DEHP enhanced RC deformability and resulted in a more "flexible" cell after prolonged storage. The fact that we observed no difference in ATP levels with different concentrations of DEHP, showed that DEHP exerted its effect directly on membrane flexibility and not on RC function. Decreased deformability is directly related to loss

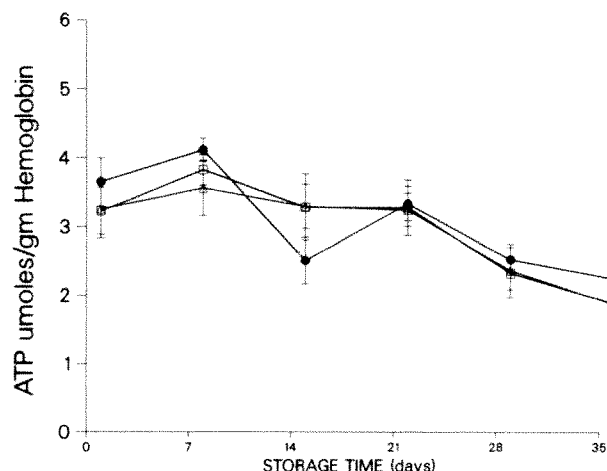


Fig 4. ATP concentration in RCC—variation with storage time in three types of storage containers: PL146, Δ; PL732 plus DMSO, □; PL732 plus DMSO and DEHP, ●.

of membrane surface area, which is due to the altered cholesterol to lipid ratio.⁶ The protection by DEHP may be due to the incorporation of the very hydrophobic DEHP into the area that was vacated by the lipid, alternatively the DEHP may help by bridging the asymmetric bilayers. By a better understanding of the mechanism of this DEHP protection, it might be possible to introduce some other stabilizing lipophilic compound that would not be as toxic as DEHP.

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CONCISE REPORT

Marrow Transplantation for Children With Acute Lymphoblastic Leukemia in Second Remission

By Jean E. Sanders, E. Donnell Thomas, C. Dean Buckner, and Kristine Doney

Fifty-seven children between the ages of 3 and 17 years with acute lymphoblastic leukemia (ALL) in chemotherapy-induced second bone marrow remission were given cyclophosphamide, total body irradiation, and bone marrow transplants from HLA-matched donors. Sixteen died of transplant-related complications. Eighteen relapsed between 56 and 833 days after transplantation, and 16 died of

leukemia. Two survive in remission off treatment following chemotherapy. Twenty-three survive in continuous remission from 1.4 to 10.4 years after transplantation and the actuarial analysis shows disease-free survival of 40% with a plateau extending from 2.5 to 10.4 years.

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TWO RECENT REPORTS concluded that chemotherapy for children with acute lymphoblastic leukemia (ALL) in second remission results in disease-free survival equal to marrow transplantation and suggest that marrow transplantation should not be recommended for these patients.^{1,2} It seemed worthwhile, therefore, to review the experience of the Seattle transplant team with marrow grafting for similar children

MATERIALS AND METHODS

Fifty-seven consecutive children <18 years of age with ALL in second marrow remission received marrow transplants from HLA identical siblings at the Fred Hutchinson Cancer Research Center between February 1973 and July 1985 and were analyzed as of January 1, 1987. Initial marrow relapse occurred while receiving chemotherapy in 52 patients and after chemotherapy had been discontinued in five patients.

Preparation for engraftment consisted of two doses of intrathecal methotrexate (MTX) (3 to 12 mg), cyclophosphamide (60 mg/kg for two doses), and total body irradiation (TBI). Nine patients received 9.2 Gy, 34 received 10.0 Gy in a single exposure, and 14 received 12.0 to 15.75 administered as 2.0 Gy/d or 2.25 Gy/d for six or seven consecutive days. Marrow was infused immediately following the last dose of TBI. Prophylaxis for graft-v-host disease (GVHD) consisted of MTX in 55 patients and cyclosporine in two.^{3,4} The grading and treatment of acute and chronic GVHD have been described elsewhere.^{3,5} Protocols and consent forms were approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center, and all patients gave informed consent.

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RESULTS

All 57 patients engrafted as shown by rising peripheral blood counts and by marrow studies, which was confirmed in 29 patients by blood genetic markers.

Fourteen patients, five with and nine without acute GVHD, died in the first 102 days, one of infection, seven of cytomegalovirus pneumonia, one of *Pneumocystis carinii* pneumonia, one of idiopathic pneumonia, two of leukoencephalopathy,⁶ one of GVHD, and one of immunoblastic sarcoma. One patient died 409 days after transplant with complications of chronic GVHD, and one patient died 470 days after transplant of leukoencephalopathy.

Eleven patients developed grade II to IV acute GVHD. Chronic GVHD occurred in eleven patients, ten with de novo onset and one after grade II acute GVHD. Seven of these 11 responded completely to immunosuppressive therapy and have Karnofsky scores of 100% off treatment and four developed recurrent leukemia.

Eighteen patients relapsed after transplantation (Fig 1). Sixteen patients died of leukemia. Two of the relapsed patients achieved a remission with chemotherapy, were treated over a 2- to 3-year period, and are in remission 6 months after discontinuing chemotherapy. Relationship of prognostic factors to relapse is shown in Table 1.

Figure 1 shows the probability of disease-free survival with a plateau at 40% extending from 2.5 to 10.4 years.⁷ Duration of first remission, WBC at diagnosis, history of extramedullary relapse, or GVHD did not influence probability of disease-free survival (Table 1).

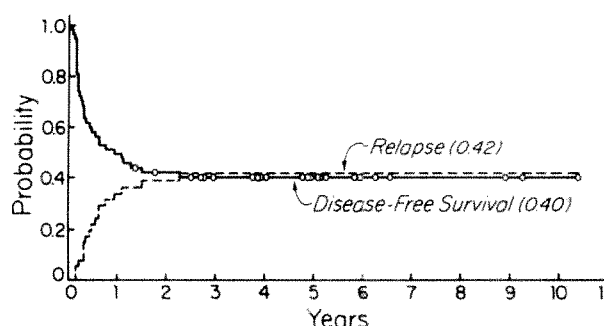


Fig 1. Kaplan-Meier product limit estimates for probability of disease-free survival and of relapse. The open circles indicate living patients.

Table 1. Relationship of Prognostic Factors to Outcome

Prognostic Factor	No. of Patients	Probability of Disease-Free Survival*	Significance†	Probability of Relapse	Significance
WBC at diagnosis					
<50,000/mm ³	38	0.42		0.38	
>50,000/mm ³	14	0.29	0.23	0.54	0.24
Extramedullary disease					
Yes	35	0.40		0.43	
No	22	0.40	0.89	0.39	0.83
Length of first remission					
<1 yr	11	0.45		0.48	
1-3 yr	31	0.37		0.42	
>3 yr	15	0.53	0.77	0.36	0.50
GVHD					
None	36	0.48		0.52	
Acute	11	0.67		0.33	
Chronic	10	0.80	0.25	0.20	0.20

*Kaplan-Meier product limit estimates.⁷

†Differences calculated by log-rank test.

DISCUSSION

Recent reports suggest no difference in survival between marrow transplantation from matched sibling donors and chemotherapy for children with ALL who achieve a second remission. Chessells et al¹ reported 53 children in second remission including 40 treated with chemotherapy and 13 with a donor who were eligible for a marrow transplant. However, only ten patients were actually transplanted but all 13 were included in the transplant group analysis. After 1 to 5.5 years of follow-up the actuarial analysis showed a 5-year disease-free survival of 35% for the transplanted patients and 20% for the chemotherapy recipients, a difference that was not statistically significant. Buchanan et al² reporting for the Pediatric Oncology Group described 198 patients who achieved a second remission and were treated with further chemotherapy. Twenty-four percent survived 18+ months. Twenty-eight similar patients given an allogeneic marrow graft showed a 24% survival at 18+ months. The short follow-up and the high incidence (50%) of toxic deaths in the marrow transplantation group make interpretation of these results difficult. Rivera et al,⁸ also reporting for the Pediatric Oncology Group, described the results of intensive chemotherapy for children with ALL who relapse. Five of 31 patients (16%) who achieved a remission were alive without disease after 38 to 44 months. All five had an initial marrow remission of more than 18 months.

The Kaplan-Meier probability of disease-free survival for the 57 children in this report shows a plateau at 40% extending from 2.5 to 10.4 years. Since more than half of the survivors are now more than four years posttransplant, it is reasonable to expect that the majority are cured of their

disease. Although our patients were not part of a prospective study, the spectrum of risk factors shows that they were not selected as good risk patients.

Three single institution prospective studies of children in second remission of ALL compared the results of marrow transplantation with chemotherapy. Of the 14 children reported by Johnson et al,^{9,10} four are disease-free 7 to 10 years after transplantation while in second remission. All 21 patients treated with chemotherapy after achieving second remission relapsed and died by 3 years. The University of Minnesota actuarial relapse-free survival for 15 children treated with a marrow transplant was 33% at 36 months compared with 5% for 23 patients treated with chemotherapy.¹¹ The Genova group reported an actuarial probability of being in continuous remission at 2 years of 58% for 17 children who received marrow transplantation compared with 18% for 19 children receiving chemotherapy.¹² A review by Butturini et al suggested that marrow transplantation was superior to chemotherapy for those children who relapsed within 18 months of first remission.¹³

The therapy of childhood ALL is improving both with chemotherapy and with marrow transplantation, but we wish to emphasize the importance of long-term follow-up before meaningful comparisons can be made. Prospective studies that evaluate outcome for all children who achieve second remission are needed to determine the optimum treatment for these children, since matched sibling donors are available for only one-third of the potential recipients. Based on the present long-term data, we conclude that all children with a suitable donor who achieve a second remission should be offered a transplant.

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CONCISE REPORT

Identification of a Restriction Fragment Length Polymorphism Involving the Oncogene ETS-1 on Chromosome 11q23

By Paul D. Savage, Curtis A. Hanson, and John H. Kersey

Twenty four samples of DNA from 23 unrelated individuals were analyzed for the presence of a novel restriction fragment length polymorphism (RFLP) involving the proto-oncogene ETS-1 at an *Xba* I site. Four samples from unrelated individuals lacked an *Xba* I site, giving rise to a longer restriction fragment detectable by Southern analysis; two samples were from normal tissue, and two were from acute myelogenous leukemic blasts. Thus, no association could be found between the RFLP and disease among

the individuals studied. Pedigree analysis of another cohort demonstrated Mendelian inheritance consistent with a somatic polymorphism. The practical applications of RFLP analysis in clinical and research settings, and the usefulness of this *Xba* I RFLP in the study of hematologic malignancies because of its location in 11q23, are discussed.

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VARIATION IN PHENOTYPIC characteristics between individual members of a species is often the result of minor base sequence differences between the genes responsible for that phenotype. Those differences that occur at particular restriction endonuclease sites are detected following restriction endonuclease digestion and Southern analysis by the presence of fragments of varying lengths. These fragment length differences are referred to as restriction fragment length polymorphisms (RFLPs), and their analysis has been shown to be useful for a number of studies, including assistance in the mapping of genes to certain regions of the genome,¹ serving as markers for certain diseases or phenotypes,² and determination of engraftment in allogeneic bone marrow transplantation.³

Many types of malignancies, both hematologic and nonhematologic, are associated with cytogenetic abnormalities of chromosome 11 band q23 (11q23).⁴ A number of genes have been mapped to this area, including the proto-oncogene ETS-1.⁵ ETS-1 has been of interest because proto-oncogene activation, which occurs as a result of translocations, deletions, or amplification, can be important in the pathogenesis of these malignancies.

Using the genomic ETS-1 probe described by de Taisne et al.,⁵ we have looked at many cases of acute lymphocytic leukemia (ALL), acute nonlymphocytic leukemia (ANLL), and a leukemic cell line with a known translocation of 11q23.⁶ While we have not found any evidence of ETS-1 abnormalities to date, we have identified an RFLP involving ETS-1 that we wish to report here. This RFLP could be important in future studies of ETS-1 and gene mapping of the 11q23 region.

MATERIALS AND METHODS

Leukemic tissues used in this study included excess peripheral blood and/or bone marrow obtained during diagnostic work-up or relapse studies; materials were either fresh or quick frozen in DMSO and stored in liquid nitrogen. Leukemias were analyzed by routine histopathologic studies and cell marker analysis and classified according to the French-American-British (FAB) system. Cell lines were representative of human lymphoid and myeloid lineages and were used because they represented leukemias that are cytogenetically normal at 11q23; the cell line RS(4;11) was also used because it contains a known t(4;11)(q21;q23) that was described elsewhere.⁶ Normal DNA was obtained either from placentas or excess bone marrow (obtained during harvesting for bone marrow transplantation); the cell line HF-26 was also used as a source of normal DNA.

The use of all human material was approved by the University of Minnesota Committee on the Use of Human Subjects in Research; the methods and facilities used followed NIH guidelines for recombinant DNA studies.

Genomic DNA was extracted from tissue using the nuclei isolation technique as described by Bahkshi et al.⁷ Ten micrograms were then digested with the indicated enzyme under conditions as outlined by the enzyme manufacturer for four hours (Bethesda Research Laboratories, Gaithersburg, MD). The restriction fragments were size fractionated on a 0.8% agarose gel, then transferred to an activated nylon membrane (Zetabind; AMF Cuno, Meriden, CT) with 20X SSC (standard saline citrate) using the Southern technique.⁸

The probe used in this study is the 5.4 kb *Eco* RI human genomic ETS-1 probe described by de Taisne et al.⁵ Radiolabeled probes were synthesized to high specific activity using the random hexamer primer method⁹ and ³²P-dCTP.

Hybridization was performed for 48 hours in the presence of 50% formamide at 42°C. Posthybridization was performed at high stringency, the final washes being done with 0.1X SSC, 0.1% SDS at 60°C, for one hour. The blots were air dried for six hours, and exposed to Kodak XAR-5 film (Rochester, NY) at -70°C for one-half to three days.

RESULTS

In the course of experiments to evaluate possible ETS-1 rearrangements in leukemias, we found a nongermline restriction fragment on an *Xba* I digest of cells from a patient with ANLL with FAB-M4 morphology. Since multiple other enzyme digests revealed germline configuration, we sought to determine whether this was an RFLP or a pathophysiologic point mutation. In order to evaluate this question, DNA

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was studied from a number of normal individuals. A Southern blot of an *Xba* I digest of total genomic DNA from 19 unrelated individuals studied with the ETS-1 genomic probe is shown in Fig 1. This probe hybridizes to restriction fragments of 6.6 kb and 1.15 kb in length in most individuals; however, four samples demonstrated a 2.4 kb restriction fragment in addition to the other two. The presence of only one additional band (2.4 kb) in the four individuals, combined with a probe size of 5.4 kb, can best be explained by loss of the *Xba* I site flanking the 1.15 kb fragment, with extension of this fragment to the next *Xba* I site, 1.25 kb away. Thus, the 2.4 kb fragment and the 1.15 kb fragment appear to be alleles. In no case did we find a homozygote who lacked the 1.15 kb fragment. This appears to represent an RFLP, in that germline configuration is found on digestion of these DNAs with other enzymes (at least three [*Bam* HI, *Eco* RI, *Hind* III], and up to eight in some cases [including *Bgl* II, *Kpn* I, *Pst* I, *Sal* I, and *Sst* I]). Further evidence that this is an RFLP resulted from demonstration that it is transmitted in a Mendelian co-dominant inheritance pattern on analysis of family members (data not shown); here, too, no homozygote for the 2.4 kb allele was found. Thus, the two alleles may be represented as follows:

Allele no.	Size (kb)	Frequency
1	1.15	0.913
2	2.4	0.087

DISCUSSION

RFLPs are useful as unique markers for certain regions of the genome; as such, they are useful to both laboratory and clinical investigators for a variety of studies. We have described a new RFLP that involves an *Xba* I site within the ETS-1 locus; it is interesting to note that the size of this other restriction fragment is approximately the same as that of the rearranged *Xba* I fragment reported by Rovigatti et al.¹⁰ This site is important as 11q23 is involved more than any other region of the genome in ANLL⁴; abnormalities of 11q23 are also reported for ALL, non-Hodgkin's lymphoma (NHL) (both 1° and 2°), solid tumors,⁴ congenital disorders,¹¹ and most recently, as the most common site of involvement when progressive cytogenetic abnormalities are seen in childhood acute leukemia.¹²

The role of the proto-oncogene ETS-1 in the pathogenesis of many of the above malignancies is unclear, although there is evidence that the ETS-1 proto-oncogene moves to chromosome 4 in at least some translocations associated with these leukemias.¹³ We have not to date demonstrated rearrangement in the cases that we have studied. The significance of this RFLP that we report here is not clear. Proto-oncogene activation may occur following point mutation, as has been demonstrated with two other proto-oncogenes,^{14,15} and it is interesting to note that we did not detect a homozygote for allele no. 2, although this latter point is most likely secondary to the small sample size. The significance of an ETS-1 point mutation can only be definitively answered once the normal ETS-1 cDNA sequence, as well as the sequences of the

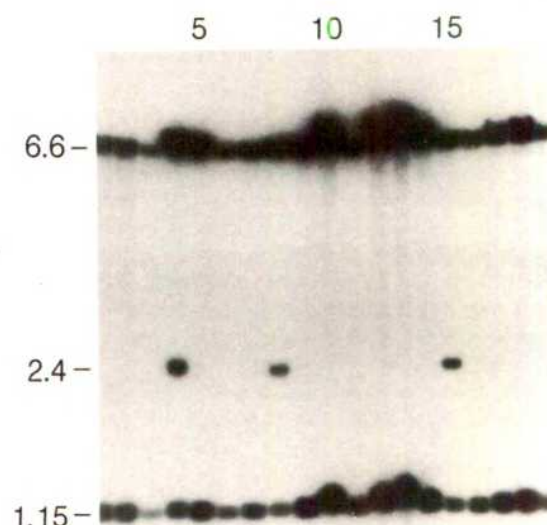


Fig 1. Southern blot of 10 µg genomic DNA digested with *Xba* I, and probed with the 5.4 kb *Eco* RI genomic ETS-1 probe. Numbers at the top are lane numbers; numbers on left are restriction fragment sizes, in kilobases (kb). Lanes 1 through 4, normal controls; lanes 5 through 7, leukemic cell lines; lane 8, "normal" cell line HF-26; lanes 9 through 19, patients with acute myelogenous leukemia (AML). The RFLP is demonstrated in lanes 4, 8, and 15. The variation in band intensity from lane to lane is secondary to unequal loading of DNA.

RFLPs in both normal and pathologic tissues, has been determined. Care must be taken in analyzing this region of DNA with *Xba* I, or any restriction endonuclease whose site may partially overlap the *Xba* I site, until the exact base changes are known, since the variant length restriction fragments can be mistaken as evidence for gene rearrangement if further analysis with other enzymes is not performed.

This RFLP may be useful as a marker for a predilection to a disease or group of diseases. Our data do not support nor refute this at present, although the numbers of normal specimens and malignant subtypes are small; further studies involving larger numbers of cases are needed in order to address this issue.

We feel it is of interest to note that this *Xba* I RFLP is the fourth RFLP that has been identified within the ETS-1 locus; the other three are *Ava* II¹⁶ and two for *Sst* I.^{16,17} All four are detectable with the same probe, and three RFLPs (*Ava* II, *Sst* I, *Xba* I) lie within a few kilobases of each other.¹⁶ To date no other RFLPs have been reported within 11q23 that are not detected by ETS-1 probes.¹⁶

ACKNOWLEDGMENT

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Localization of the G-CSF Gene on Chromosome 17 Proximal to the Breakpoint in the t(15;17) in Acute Promyelocytic Leukemia

By Ruth N. Simmers, Lorna M. Webber, M. Frances Shannon, O. Margaret Garson, Gordon Wong, Mathew A. Vadas, and Grant R. Sutherland

The human granulocyte-colony stimulating factor gene (G-CSF) is localized at 17q11.2-q21, the region of one of the breakpoints in the 15;17 chromosome translocation specific for acute promyelocytic leukemia (APL). As G-CSF induces differentiation and loss of tumorigenicity in myeloid leukemic cells or cell lines, it was possible that the translocation in APL involved the DNA of the G-CSF coding region or its regulatory region. In situ hybridization to chromosomes with the t(15;17) from patients with the APL

translocation using a G-CSF cDNA clone revealed that the coding region of this gene is proximal to the t(15;17) breakpoint on chromosome 17. Southern analysis of DNA from patients with the APL translocation showed no differences in hybridization between normal and leukemic cells. These results indicate that the G-CSF coding sequence is not disrupted by the chromosomal rearrangement characteristic of APL.

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THE SPECIFIC CHROMOSOME rearrangement observed in the malignant cells of patients with acute promyelocytic leukemia (APL) involves chromosomes 15 and 17, but breakpoints in the translocation remain controversial, with that on chromosome 17 being localized variously from 17q11.2 to 17q22.¹⁻⁴ The gene that encodes for human granulocyte-colony stimulating factor (G-CSF) has been mapped to the region 17q11.2-21 by in situ hybridization.⁵ G-CSF normally stimulates progenitor cells to proliferate and differentiate into neutrophils,^{6,7} and will also stimulate leukemic cells or cell lines to differentiate into mature neutrophils.⁸⁻¹¹ It is therefore possible that the 15;17 translocation in APL involves either the G-CSF gene or its regulatory region.

As the specific chromosome rearrangement in APL divides 17q into two regions, it is of interest to know to which of these regions G-CSF maps and whether the translocation alters the in situ hybridization patterns. We have studied the t(15;17) in two patients with APL using in situ hybridization with the G-CSF probe and in four patients using Southern analysis.

MATERIALS AND METHODS

DNA studies. Bone marrow cells from patients with APL were cultured for 24 hours before being synchronized with 0.1 μ mol/L fluorodeoxyuridine.¹² The block was released by the addition of 20 μ mol/L bromodeoxyuridine and the chromosomes harvested 8½

hours later after 30 minutes' exposure to colcemid. These chromosomes were banded and photographed prior to in situ hybridization.

This was carried out using a cDNA clone consisting of the entire coding region of the G-CSF gene in the plasmid pXMT2, to these bone marrow and to normal male lymphocyte chromosomes as described by Simmers et al.⁵ Kodak NTB-2 (Rochester, NY) and Ilford L4 (Essex, UK) nuclear research emulsions were used for autoradiography.

Southern analysis was performed, using the G-CSF probe, to DNA from normal and leukemic cells of four patients with APL and the t(15;17). (DNA was kindly supplied by Professor A. Morley and Dr L. Ashman.) The DNA was cut by four restriction enzymes: *Eco* RI, *Bam* HI, *Bgl* II, and *Hind* III.

Patients. Two patients with APL were studied using in situ hybridization. Patient ML was a 72-year-old woman who presented with APL (M3) in June 1986. She died from uncontrollable bleeding shortly after admission. The leukemic cell karyotype showed the typical 46,XX,t(15;17)(q22;q21) in 95% of metaphases examined.

Patient SD was a 15-year-old boy who presented with APL in November 1984. The leukemic karyotype was 46,XY,t(15;17)(q22;q21),i(17q-); an isochromosome of the partially deleted 17q is a recognized variant of the t(15;17) in APL.¹³ The patient achieved remission, but subsequently relapsed on several occasions and although additional chromosome abnormalities were found at relapse, the t(15;17),i(17q-) was always present, with approximately 20% of metaphases having an additional i(17q-). The patient died 21 months after diagnosis.

RESULTS

Examination of 25 normal metaphases probed for G-CSF revealed significant label on the long arm of chromosome 17: 22 out of 108 silver grains (20%) were seen over this region ($P < 10^{-5}$ using cumulative Poisson probabilities), indicating that the probe had hybridized to G-CSF on 17q. Chromosomes from 121 metaphases of patient ML had 16 silver grains (6.4% of a total of 249 silver grains over all chromosomes; $P < 10^{-5}$) over the region 17cen to 17qter on the normal chromosome 17; 22 (8.8% of 249; $P < 10^{-5}$) over 17cen to 15qter on the derived chromosome 17; and four (1.6% of 249; NS) over both 15q15 to 15qter and 15q15 to 17qter on the normal and derived chromosomes 15, respectively. Chromosomes from 130 metaphases of patient SD had 14 silver grains (4.9% of a total of 287 silver grains over all chromosomes; $P < 10^{-5}$) over 17cen to 17qter on the normal chromosome 17; 44 (15.3% of 287; $P < 10^{-5}$) over the isolated 17q; and four (1.4% of 287; NS) and five (1.7% of 287; NS) over 15q15 to 15qter and 15q15 to 17qter on the normal and derived chromosomes 15, respectively. In sum-

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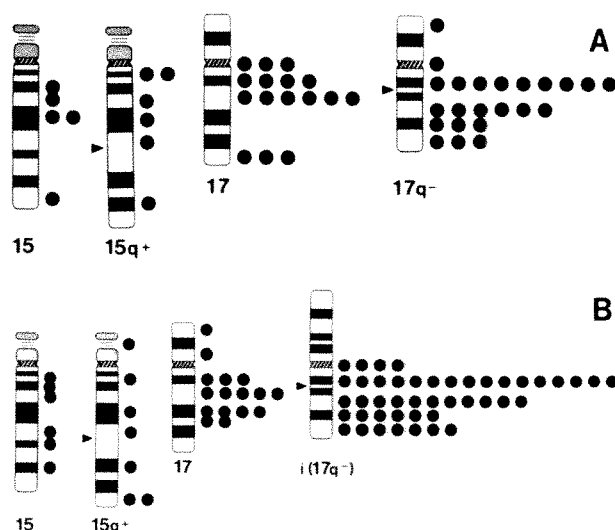


Fig 1. The distribution of silver grains scored over the normal and derived chromosomes 15 and 17 of (A) patient ML; and (B) patient SD. Note in (B) that there are two copies of 17q⁻ in the i(17q⁻), but as it is not possible to differentiate morphologically between the two arms of the isochromosome, the label is represented on only one arm; also note that about 20% of metaphases had an extra copy of this chromosome. The regions involved in translocation are indicated by arrows.

mary, metaphases from both patients showed significant labeling of the derived and normal chromosomes 17, but not of the derived chromosomes 15 (Fig 1). These results indicate that the G-CSF coding region is proximal to the breakpoint on chromosome 17 and therefore has not been moved in the translocation.

Southern analysis failed to reveal rearrangement of the coding region of the G-CSF gene.

DISCUSSION

The chromosome 17 breakpoint of the translocation in APL cells has been located variously from 17q11.2 to 17q22.¹⁻⁴ This region overlaps with the localization of G-CSF at 17q11.2-21 recently described by Simmers et al.⁵ Thus, while the location of the G-CSF gene has not been defined more precisely by the present results, the position of the coding region with respect to the breakpoint and its probable noninvolvement in the translocation have been revealed.

The oncogene *c-erbA* has also been mapped proximal to the chromosome 17 breakpoint in APL¹⁴⁻¹⁶ and no rearrangement of the *c-erbA* gene resulting from this translocation has been detected.¹⁷ In a case of undifferentiated acute leukemia found to have a t(17;21)(q21-q22;q22), the *c-erbA* gene was localized proximal to this breakpoint at 17q21-22.¹⁴ In addition, the nerve growth factor receptor gene (*NGFR*) has been mapped distal to this breakpoint at 17q12-22.¹⁸ Although cytogenetically similar, the point of translocation in this undifferentiated leukemia is not necessarily the same at the DNA level as in the t(15;17) in APL, so it cannot be assumed that *NGFR* is actually distal to the breakpoint in APL. No rearrangement of the *NGFR* gene was detected in chromosomes with the t(17;21) or the APL translocation.¹⁸

As the G-CSF probe used for in situ hybridization in our studies contains the entire coding region of the G-CSF gene, the results indicate that these coding regions are proximal to the site of rearrangement in APL. However, this information does not exclude the possibility of the rearrangement occurring through a region of DNA flanking the coding sequence, affecting the regulation of G-CSF production.

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Human Recombinant Multilineage Colony Stimulating Factor (Interleukin-3): Stimulator of Acute Myelocytic Leukemia Progenitor Cells In Vitro

By Ruud Delwel, Lambert Dorssers, Ivo Touw, Gerard Wagemaker, and Bob Löwenberg

Acute myeloid leukemia colony forming cells (AML-CFU) require the addition of colony stimulating factors (CSFs) for in vitro proliferation. Recently, we isolated a human recombinant multilineage CSF (hMulti-CSF). We investigated the ability of hMulti-CSF to stimulate AML clonogenic cells in seven patients in direct comparison with the effects of human granulocyte CSF (hG-CSF), human granulocyte-macrophage CSF (hGM-CSF), and feeder leukocytes. We show that hMulti-CSF is an efficient stimulator of AML colony formation in four of seven cases. In these patients, hGM-CSF was also capable of stimulating AML colonies in vitro. In two of seven cases hMulti-CSF appeared to be a weak stimulus of AML-CFU proliferation. In these latter two cases, however, hG-CSF and in one case hGM-CSF effectively stimulated AML-CFU growth. In one patient none of the hCSFs, either alone or in combination, induced AML colony formation, whereas AML colonies consistently appeared in the phytohemagglutinin (PHA) leukocyte

feeder assay. This finding suggests that PHA stimulated leukocytes produce components other than the tested hCSFs that may have a role in the proliferation of AML cells in vitro. Multi-CSF, like hGM-CSF, revealed a limited capacity to induce progressive maturation during AML colony growth, ie, not beyond the promyelocytic stage. On the other hand, in one case, hG-CSF stimulated the growth of AML colonies containing (meta)myelocytes and granulocytes. We conclude that hMulti-CSF is a regulator of AML-CFU proliferation in a significant number of cases. The patterns of responsiveness of AML precursors to the three hCSFs in different patients show a striking variability, which may indicate that AML-CFU are the neoplastic representatives of normal bone marrow progenitors at different stages of maturation and with distinct CSF requirements.

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THE COLONY STIMULATING factors (CSFs) comprise a group of hormones that stimulate the proliferation and differentiation of hematopoietic precursor cells in vitro.¹ Recently, we isolated a human multilineage CSF (hMulti-CSF) cDNA clone from a cDNA library of phorbol ester and lectin stimulated human leukocytes.² This hMulti-CSF cDNA was identified by virtue of sequence homology occurring within the 3' terminal untranslated region of mouse interleukin-3 (mIL-3). Protein homology between hMulti-CSF and mIL-3 appeared to be low (28%).² Expression of this hMulti-CSF cDNA clone in mammalian cells resulted in the production of a CSF that stimulates the in vitro proliferation of normal hematopoietic clonogenic cells, ie, granulocytic, erythroid, and multipotent progenitors.² This hMulti-CSF is very similar to a gibbon Multi-CSF described by Yang et al³ with respect to DNA and protein sequence as well as biologic activity. In the present study we analyzed the role of this factor in the proliferation of acute myelocytic leukemia clonogenic cells (AML-CFU) in vitro. Since two other human CSFs, ie, granulocyte-macrophage CSF (hGM-CSF) and granulocyte CSF (hG-CSF), had become available earlier by recombinant DNA technology and had been demonstrated to stimulate AML colony growth in vitro,^{4,9} we examined the effect of hMulti-CSF on AML progenitors in culture in direct comparison with those hCSFs.

MATERIALS AND METHODS

AML blast cells. The blasts from seven adult patients with AML were studied. The AML cases were classified according to the criteria of the French-American-British (FAB) committee.^{10,11} AML blasts were purified from bone marrow (patients no. 1, 4, and 6) or blood (patients no. 2, 3, 5, and 7) using a bovine serum albumin (BSA) density gradient centrifugation. Contaminating T lymphocytes were removed from those cell samples by E-rosette sedimentation as described.^{12,13} All patients had given informed consent to donate bone marrow and blood.

Recombinant CSF preparations. Recombinant hGM-CSF and hG-CSF preparations were obtained from Genetics Institute (Cam-

bridge, MA). The structural and biological characteristics of these hCSFs have been reported in detail.^{4,9,14,15} Recombinant hMulti-CSF was produced in monkey (Cos1) cells by transfection of a eukaryote expression plasmid (pLB4) containing the hMulti-CSF cDNA.² Cos/pLB4 conditioned medium was harvested 48 to 72 hours after transfection. Conditioned medium from Cos cells transfected with a vector not containing the hMulti-CSF gene did not stimulate AML-CFU proliferation.

AML colony formation. AML colony numbers were assessed in the standard PHA leukocyte feeder (PHA l.f.) assay.^{12,13} Colonies (groups of 50 cells or more) were counted on days 7 through 9 of culture. To study the dependence of AML-CFU on the recombinant hCSFs, the preparations were added separately as well as in combination to the upper layer of the cultures while no feeder cells had been incorporated into the underlayers. After culture, the AML colony cells were mass harvested and analyzed morphologically on May-Grünwald Giemsa stained cytocentrifuge slides.

RESULTS

The optimal concentrations of the hCSFs to induce AML colony formation were determined in dose-response experiments (Fig 1). Based on these data further experiments were performed with 10% (vol/vol) hMulti-CSF, 0.1% (vol/vol)

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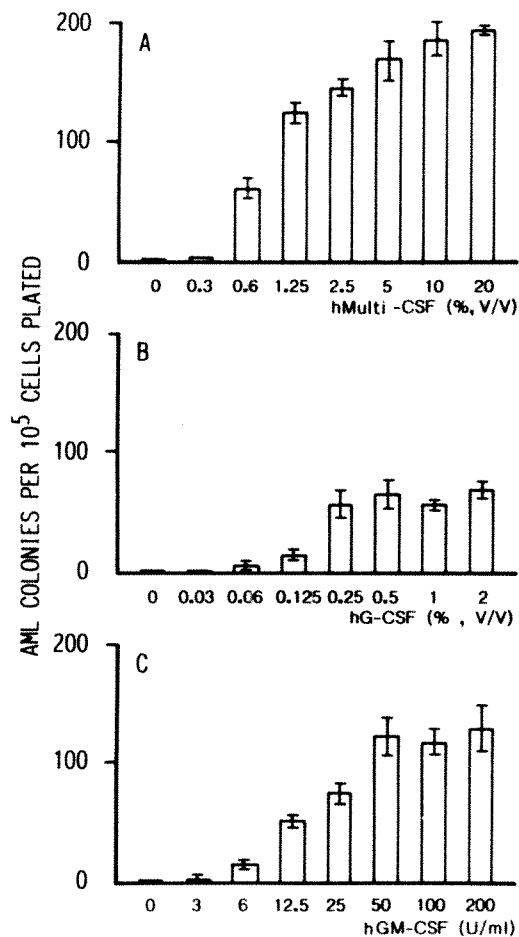


Fig 1. AML colony formation (patient no. 6) with titrated concentrations of the hCSFs. (A) hMulti-CSF, (B) hG-CSF, and (C) hGM-CSF.

hG-CSF, and 100 U/mL hGM-CSF. The effects of hMulti-CSF on AML colony formation as compared with hGM-CSF, hG-CSF, and feeder leukocytes are shown in Table 1. Without the addition of any of the stimuli, no colonies were formed. AML colony formation was stimulated efficiently by hMulti-CSF in cases no. 2, 4, 5, and 6. In patients no. 2 and 6, hMulti-CSF alone induced numbers of AML colonies that exceeded the values of the standard PHA I.f. assay. In the other two cases (no. 4 and 5) suboptimal colony formation was observed, as compared with feeder leukocyte stimula-

tion. In patients no. 1 and 3, a consistent but minimal colony response to hMulti-CSF was apparent. In one case (no. 7) hMulti-CSF failed to induce AML colonies.

The stimulatory activity of hMulti-CSF on AML-CFU proliferation paralleled that of hGM-CSF, with the exception of case no. 1, in which hGM-CSF stimulated larger numbers of colonies than did hMulti-CSF. On the other hand, hMulti-CSF and hG-CSF stimulation patterns were opposite. In the cases showing a significant colony response to hMulti-CSF (cases no. 2, 4, 5, and 6) no or considerably fewer numbers of colonies were induced by hG-CSF, whereas in the hG-CSF high responders (cases no. 1 and 3) the effect of hMulti-CSF was minimal.

The data from Table 1 indicate that in most cases more than one recombinant hCSF stimulated AML colony formation. To investigate the possibility that the different hCSFs acted on AML-CFU in an additive fashion the preparations were also tested in combination. Only in cases no. 2 and 6 was an indication of additive stimulation by hMulti-CSF and hGM-CSF obtained. In the other cases the stimulatory effect of the single most efficient stimulator was not enhanced as a consequence of using a mixture of the three hCSFs in culture.

In cases no. 1, 3, 4, and 5 it appeared that AML colonies were induced more efficiently in the PHA I.f. assay than in the cultures with the complete mixture of recombinant hCSFs. Moreover, AML-CFU from patient no. 7 did not respond to the three hCSFs, whereas significant numbers of colonies appeared in the PHA I.f. assay. These data suggest that feeder leukocytes may produce at least one other component regulating AML-CFU proliferation.

The AML colony cells of patients no. 1, 3, and 6 were harvested and analyzed morphologically to assess whether the CSFs differ in their capacity to induce maturation (Table 2, Fig 2). In these cases the colony cells from the cultures stimulated by either hMulti-CSF or hGM-CSF showed limited differentiation, ie, not beyond the promyelocytic stage. In contrast, hG-CSF-stimulated colonies from patient no. 6 contained cells that had matured towards (meta)myelocytes and granulocytes (Fig 2).

DISCUSSION

We investigated the stimulative effects of hMulti-CSF, in comparison with the effects of hG-CSF and hGM-CSF and feeder leukocytes on the proliferation and differentiation of

Table 1. Induction of AML Colony Formation

	Case No. 1 (M2)*	Case No. 2 (M1)	Case No. 3 (M1)	Case No. 4 (M4)	Case No. 5 (M2)	Case No. 6 (M1)	Case No. 7 (M1)
No addition	0	0	0	0	0	0	0
hMulti-CSF	5 ± 2†	169 ± 13	9 ± 3	22 ± 2	24 ± 9	197 ± 23	0
hG-CSF	70 ± 8	0	68 ± 17	0	0	24 ± 6	0
hGM-CSF	104 ± 13	100 ± 3	2 ± 0	12 ± 2	22 ± 0	123 ± 6	0
hMulti-CSF + hG-CSF + hGM-CSF	125 ± 11	207 ± 7	70 ± 13	26 ± 1	34 ± 4	323 ± 12	0
PHA I.f.	256 ± 23	94 ± 9	103 ± 12	50 ± 12	61 ± 13	107 ± 6	24 ± 8

The effects of hMulti-CSF, hG-CSF, and hGM-CSF on AML colony formation were studied in a modified PHA I.f. system from which feeder leukocytes were omitted. Standard PHA I.f. cultures for AML colony formation were run in parallel.

*Classification according to the FAB Cooperative Group.^{10,11}

†Colony numbers per 10⁵ cells (mean ± SD).

Table 2. Differential Counts of MGG-Stained AML Colony Cells

	Case No. 1*			Case No. 3*			Case No. 6*		
	Multi-CSF	GM-CSF	G-CSF	Multi-CSF	GM-CSF	G-CSF	Multi-CSF	GM-CSF	G-CSF
Myeloblasts	2†	1	4	25	19	18	62	58	20
Promyelocytes	92	96	84	68	68	73	26	26	17
(Meta)myelocytes	0	3	2	0	1	4	2	2	43
Granulocytes	0	0	0	0	0	0	0	0	8
Monocytes/macrophages	6	0	10	7	12	5	7	14	12
Other	0	0	0	0	0	0	3	0	0

*The purified AML samples preculture contained 51% (case no. 1), 96% (case no. 3), and 98% (case no. 6) myeloblasts. The AML sample from case no. 1 preculture contained 46% promyelocytes.

†Percentages of 250 cells counted.

AML-CFU in colony culture. Human Multi-CSF, hG-CSF, and hGM-CSF were all capable of stimulating AML colony formation in vitro, although the responses of AML-CFU of different individuals to these hCSFs were variable.

The results indicate that AML colony formation in only one of these patients was stimulated predominantly by one of the three hCSF tested (case no. 3). Usually two (cases no. 1, 2, 4, and 5) or all three hCSFs (case no. 6) were able to induce AML-CFU proliferation. In most of these patients the presence of the three hCSFs in the cultures simultaneously did not result in an increased colony response as compared with the single most active factor, suggesting that the different hCSFs acted on overlapping AML-CFU target populations. However, exceptions were patients no. 2 and 6 in whom additive effects of hMulti-CSF and hGM-CSF on AML colony formation were observed. Thus, in these cases different AML-CFU subpopulations were apparently stimulated by different growth stimuli. The data in Table 1 do not

provide evidence that the combination of the three factors can enhance colony numbers synergistically. To explore further the possibility of a synergistic action of the hCSFs, we set up cultures to which suboptimal concentrations of the different growth stimuli were added simultaneously. These studies also did not indicate synergistic stimulation of AML colony formation by these hCSFs (data not shown).

An interesting observation was that in patient no. 7 AML colony growth could not be induced by the recombinant hCSFs, whereas colonies appeared consistently in the standard PHA l.f. assay. The PHA l.f. colony culture has been an efficient assay for clonogenic AML cells,^{12,13} although the active stimulatory components of the system are probably multiple and not exactly defined. The fact that colony growth in patient no. 7 appeared only in the PHA l.f. assay could imply that components other than these hCSFs have a critical role in stimulating the proliferation of AML precursor cells. This suggestion is also supported by the fact that in

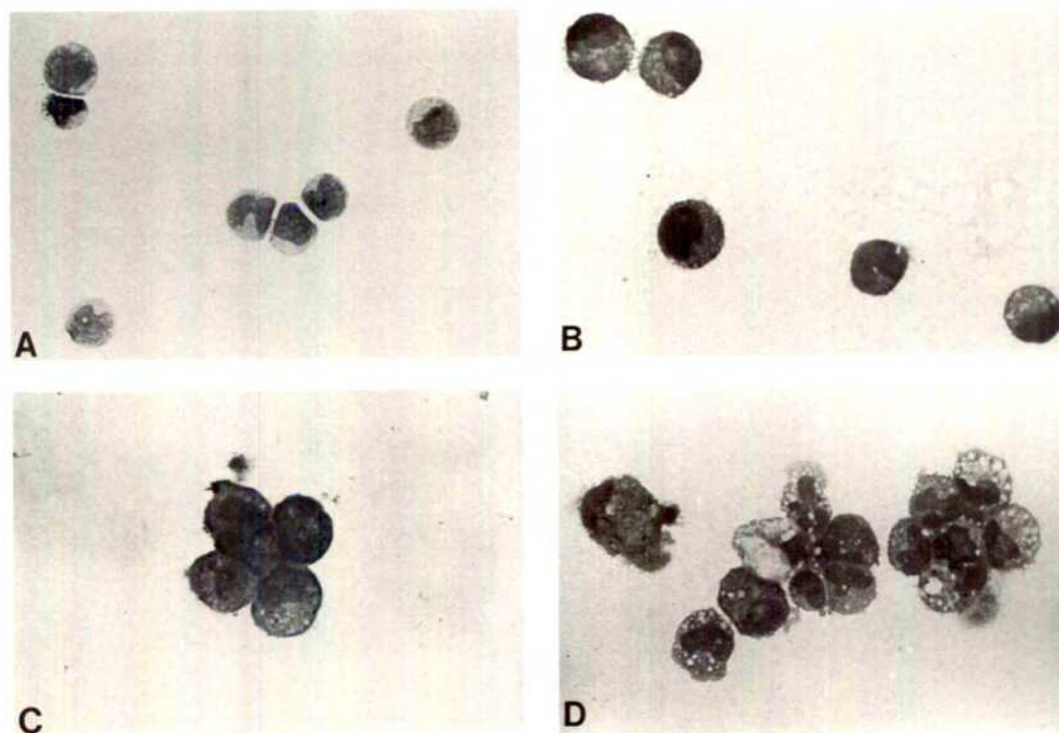


Fig 2. May-Grünwald Giemsa morphological analysis of AML cells (patient no. 6). (A) Preculture, (B) cultured with hMulti-CSF, (C) cultured with hGM-CSF, and (D) cultured with hG-CSF.

4 cases (no. 1, 3, 4, and 5) the PHA I.f. system supported the outgrowth of larger numbers of colonies than was achieved with the mixture of recombinant hCSFs.

Morphologic analysis of the AML colony cells of three patients revealed that progressive maturation occurred occasionally in the hG-CSF stimulated but not in the hMulti-CSF and hGM-CSF stimulated cultures (Table 2). Since the three factors have been reported to induce granulocytic or monocytic differentiation in normal bone marrow cultures, this could indicate that in patient no. 6, the AML blasts respond abnormally to hMulti-CSF and hGM-CSF but normally to hG-CSF, as regards the induction of maturation.^{1,2} However, at this stage, the possibility cannot be excluded that differentiation towards granulocytic end cells, observed in the normal bone marrow cultures stimulated by hMulti-CSF or hGM-CSF, is not caused by direct effects of these two factors but by endogenously produced G-CSF.

Thus, it remains unclear whether the variations of CSF responses of AML progenitors either represent leukemic characteristics of the cells or reflect the features of normal bone marrow progenitor subsets at different maturation stages with distinct CSF requirements. To discriminate between these two possibilities it will be essential to study in detail the effects of the recombinant hCSFs on highly purified normal bone marrow progenitors.

ACKNOWLEDGMENT

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CORRESPONDENCE

GAMMA INTERFERON AND APLASTIC ANEMIA

To the Editor:

Certain aspects of the published data on the role of γ -interferon (IFN- γ) in hematopoiesis in vitro and in bone marrow failure states were not emphasized in the article by Torok-Storb and co-workers.¹

Beginning with the initial observation of Kagan and colleagues,² many investigators have demonstrated that T cells from patients with aplastic anemia can suppress hematopoiesis in vitro.³⁻⁵ Bacigalupo and associates⁶ found that the supernatants of aplastic lymphocyte cultures were as effective as the cells themselves in suppression and that lectin-activated normal lymphocytes produced an inhibitory factor. These observations were important because they implicated a soluble inhibitory factor and suggested an experimental system that would avoid the allogeneic effects and imprecision that plague co-culture experiments.

We reported that the inhibitory activity in these lymphocyte supernatants was IFN- γ ;⁷ our studies have been confirmed by Murphy and colleagues,⁸ who found also that lymphotoxin was produced and acted to enhance the activity of IFN- γ , a not unexpected result given the coordinated expression of lymphokines in T cells and the known synergy between factors in inhibiting cell proliferation. Interferon production by an activated lymphocyte with the same surface phenotype as is found in the circulation of aplastic patients (CD8⁺, Tac⁺, HLA-DR⁺)⁹ has also been confirmed in other laboratories.¹⁰

In analysis of patient material, we¹¹ reported multiple abnormalities of interferon production: (a) enhanced production of interferon by lectin-stimulated mononuclear cells; (b) spontaneous production of interferon, not seen in normal cultures; (c) improvement in colony formation by aplastic bone marrow in the presence of antibodies to interferon; and (d) elevated serum levels of interferon in about 30% of patients, as determined by bioassay of fresh material by a specialist in interferon who was blinded to the clinical nature of the samples.

Abnormalities of interferon production in aplastic patients have been confirmed by other laboratories¹²⁻¹⁵ (and E.D. Zanjani, personal communication); even the abstract cited in support by Torok-Storb and co-workers concluded that "there were IFN- γ abnormalities in some patients with aplastic anemia."¹⁵ The data presented in the article by Torok-Storb and co-workers are indeed also similar to those of Zoumbos and colleagues.¹¹ First, more than one-third of patients' cells spontaneously produced interferon, in some cases to very high levels. Second, although Fig 2 (of Torok-Storb article) is plotted on a semilogarithmic scale that tends to compress the data lines, extraction of the interferon values at the plateaus and their statistical analysis shows a significant difference between lectin-stimulated interferon production by the four patients and five controls ($P < .05$ by Student's t test), again similar to the data of Zoumbos and colleagues and other investigators.

That supernatants derived from patient's cell cultures containing high concentrations of interferon did not inhibit in the study of Torok-Storb and co-workers is almost certainly due to the concomitant presence of growth factors (GM-CSF). Growth factors are antagonistic to interferon's actions in hematopoietic cell culture systems.¹⁶ More important, a major aim of our studies was to replace co-culture experiments with measurements of specific molecules and cell types. That interferon—recombinant IFN- γ —is inhibitory of hematopoietic cell proliferation has been reported by many laboratories¹⁶⁻²⁰ and in human patients treated with interferon.²¹ Torok-Storb and co-workers²² have been unable to demonstrate recombinant interferon's inhibitory effect in their cell culture system.

Finally, legitimate debate exists on the presence of interferon in serum. Our data^{7,11} were generated using the virus inhibition assay, which has recently been shown to detect lymphotoxin as well as interferon.²³ The synergy between these factors probably makes the bioassay more sensitive but less specific than the radioimmunoassay. Bioassays are not intrinsically less valuable than radioimmunoassays, however. Radioimmunoassays with monoclonal antibodies rely on detection of single or restricted numbers of epitopes, which can differ between natural and recombinant molecules²⁴ and may be lacking on molecules produced in pathologic states. In addition, molecules with very high specific activity, such as interferon, may be easier to detect with biologic than chemical techniques. Nevertheless, preliminary data obtained by Dr Leonidas Plataniotis in our laboratory (Fig 1), however, suggest that some patients with aplastic anemia will have circulating IFN- γ in their sera.

Ample laboratory data and clinical observations continue to make immune mediation of bone marrow failure an attractive hypothesis. It seems likely to me that hematopoietic depression will be effected by inhibitory molecules and that these abnormalities of the immune system reflect a viral pathogenesis, for which there is increasing evidence. The immunologic and hematopoietic systems are enormously complicated, however, and extrapolation from in vitro experiments to pathogenic mechanisms is an increasingly daunting task. Whatever interpretation eventually proves correct, it should be clear that, despite the negative title and tone, many of the differences described in the article by Torok-Storb and co-workers and in our own results, as well as those of other investigators, are more apparent than substantial.

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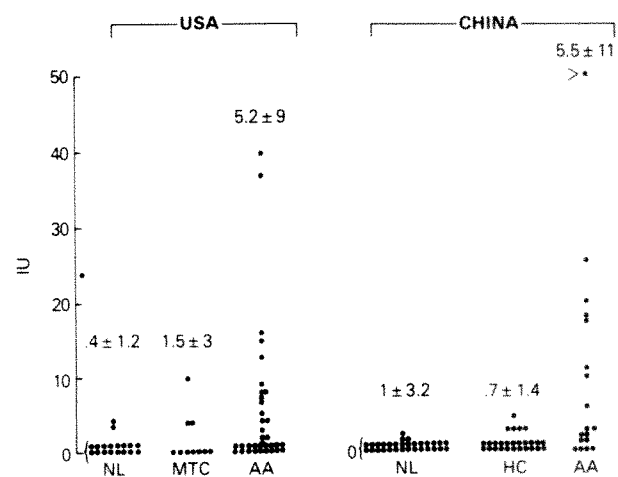


Fig 1. γ -Interferon (IFN- γ) as measured by radioimmunoassay in American and Chinese serum samples. Data were obtained without knowledge of the clinical history. Linearity of the assay was assessed with the internal standards provided by Centocor (Malvern, PA) but values were assigned by comparison with a natural IFN- γ reference standard (NIAID Gg23-901-530).

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RESPONSE

To the Editor:

In response to Dr Young's letter, I would like to reiterate three points. First, we reported that γ -interferon (INF- γ) could not be detected in serum samples obtained from 50 patients with aplastic anemia (AA). We thought this information should be published because it differed dramatically from that of Zoumbos and colleagues,¹ who detected significant levels (>10 U/mL) in 41% or 10 of 24 patients studied. Dr Young's data, published above, now indicate that 17% or 11 of 63 patients studied had serum levels >10 U/mL, which differs significantly from his previous observations.

Second, in their original publication, Zoumbos and colleagues stated that "Virtually every patient with aplastic anemia reported in this study showed abnormal interferon production in vitro." Their data indicated dramatic differences in the kinetics of INF production. Patient peripheral blood mononuclear cells (PBMCs) produced $>1,000$ U/mL of INF- γ 24 hours after lectin stimulation, and

levels continued to increase over seven days. In contrast, normal PBMC reached peak levels of $<1,000$ U/mL at day 5 and decreased to baseline levels by day 7. Our data differed dramatically from these observations. Both normal and AA PBMCs reached plateau levels of INF- γ three days post-lectin stimulation, and in both groups INF- γ levels were maintained over seven days. Clearly, in our hands the kinetics of INF- γ production did not differ between the two groups.

Third, we too believe it is noteworthy that we cannot demonstrate inhibition of BFU-E with INF- γ . This continues to be the case. Indeed, using enriched populations of precursors, we often see INF- γ augmenting BFU-E growth. Comparable results using MY 10-selected precursors have recently been reported by Strauss and associates.² These observations cannot be explained merely by the presence of GM-CSF in our cultures. Because growth of hematopoietic precursors in vitro requires growth factors, either added from exogenous sources or produced in situ, it is safe to assume that all

culture systems that support colony or burst formation contain growth factors. The explanation of these apparent and substantial differences is not obvious, but developing ways to address these issues is best served by considering both positive and negative data.

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ERRATA

In the article by Viskup, Tracy, and Mann entitled "The Isolation of Human Platelet Factor V," which appeared in the April 1987 issue (Volume 69, pp 1188-1195), there were mistakes in the legend to Figure 3. The correct legend appears below:

Fig 3. Immunoblot of human platelets drawn directly into protease inhibitors and washed as described. The primary antibody was burro antihuman factor V followed by ¹²⁵I-Protein A. Lanes: (a) washed platelets (5×10^7 platelets) lysed with glacial acetic acid; (b) washed platelets (5×10^7 platelets) lysed with detergent (SDS); (c) human plasma (7.5 uL); (d) human serum (7.5 uL).

In the article by Kelleher et al entitled "Synergism Between Recombinant Growth Factors, GM-CSF and G-CSF, Acting on the Blast Cells of Acute Myeloblastic Leukemia," which appeared in the May 1987 issue (Vol 69, pp 1498-1503), there was an error in the legend for Figure 2. The correct legend appears below:

Fig 2. A photomicrograph of blast colonies formed with different growth factors. (A) GM-CSF alone at a maximum stimulatory concentration of 2 nmol/L. (B) G-CSF alone at maximum stimulatory concentration of 200 U/mL. (C) 10% HTB-9 CM. (D) A mixture of GM-CSF and G-CSF, both at maximum stimulatory concentrations.

In the article by Sugarman, Devine, and Rosse entitled "Structural and Functional Differences Between Decay-Accelerating Factor and Red Cell Acetylcholinesterase," which appeared in the September 1986 issue (Vol 68, pp 680-684), a mathematical error was consistently made in Table 1. The correct table appears below:

Table 1. The Effect of Antibodies to AChE or DAF on the Activity of AChE

Normal RBC Pretreated With	AChE Activity (moles hydrolyzed/min/cell $\times 10^{-15}$)
Anti-AChE (AE-2)	0.53
P3 control*	0.73
Rabbit anti-DAF	0.93
Normal rabbit serum	0.70

*Monoclonal antibody lacking reactivity with RBC.

ANNOUNCEMENTS

THE THIRTEENTH CONGRESS OF THE EUROPEAN SOCIETY FOR MEDICAL ONCOLOGY (ESMO)

**October 30–November 1, 1988
Lugano, Switzerland**

The program contains two key-note lectures, an award-lecture for an outstanding young investigator, and nine sessions with proffered papers and posters, followed by a review. The first day will be devoted to satellite symposia and to the educational program.

For further information, contact: F. Cavalli, MD, Division of Oncology, Ospedale San Giovanni, 6500 Bellinzona, Switzerland.

ELEVENTH ANNUAL CANCER SYMPOSIUM SEVENTH ANNUAL CANCER SYMPOSIUM FOR NURSES

October 19-21, 1987

Sheraton Harbor Island Hotel East, San Diego, CA

Sponsored by Scripps Memorial Hospital. For the Eleventh Annual Cancer Symposium, the registration fee is \$350; accreditation is 21 hours Category I. For the Seventh Annual Cancer Symposium for Nurses, the registration fee is \$275; accreditation is 23.5 contact hours.

For further information, contact: Nomi Feldman, Conference Coordinator, 3770 Tansy, San Diego, CA 92121. Telephone: (619) 453-6222.

NEW SOLUTIONS TO OLD PROBLEMS IN SURGICAL PATHOLOGY

October 5-7, 1987

Bethesda, MD

The three day program will address difficult or evolving areas of surgical pathology, and will focus on areas that often pose diagnostic difficulty. New techniques applicable to the general surgical pathology laboratory will be presented, and clinico-pathologic correlations of morphologic diagnoses will be stressed. Participants will receive a syllabus containing lecture outline material and references. The program is designed for pathologists at the senior resident and practitioner levels. Twenty-one (21) hours AMA Category I credit.

For further information or application, please call or write: Surgical Pathology, c/o FAES, National Institutes of Health, Bldg 10, Room B1-L-101, Bethesda, MD 20892. Telephone: (301) 496-7975.

AMERICAN SOCIETY OF HEMATOLOGY
TWENTY-NINTH ANNUAL MEETING

December 5-8, 1987

Sheraton Washington/Omni Shoreham Hotels
Washington, DC

The Twenty-Ninth Annual Meeting of the American Society of Hematology will be held at the Sheraton Washington and Omni Shoreham Hotels, Washington, DC, December 5-8, 1987. Programs by the Education Committee and the Scientific Subcommittees will be held on Saturday, December 5, and Sunday, December 6. The Presidential Symposium will be Tuesday morning, December 8. Four mini-plenary sessions will be held on Tuesday afternoon. Selected papers submitted to the meeting will be presented either in oral or poster sessions on Sunday, December 6, Monday, December 7, and Tuesday, December 8.

The abstract deadline for contributed papers is September 1, 1987. Abstract forms may be obtained from SLACK, Inc (address below). Subsequent issues of BLOOD will contain details of the Educational and Subcommittee Programs and the Presidential Symposium, in addition to information about registration for the meeting. Preregistration material will be sent early this fall to members. If you do not receive your meeting information by October 6, 1987, please contact the Registration Supervisor at SLACK, Inc, 6900 Grove Rd, Thorofare, NJ 08086-9447, (609) 848-1000. Nonmembers wishing to receive preregistration information and forms should also contact SLACK, Inc.

AMERICAN SOCIETY OF HEMATOLOGY
29TH ANNUAL MEETING

December 5-8, 1987

ADVANCE REGISTRATION

NAME _____ STREET _____

CITY _____ STATE _____ ZIP _____

NAME OF SPOUSE (if attending meeting) _____

American Society of Hematology Members, \$30.00 (A) _____ Honorary or Emeritus Members, no fee (B) _____ Council on Thrombosis Members, \$30.00 (C) _____ Nonmembers, \$55.00 (D) _____ *Nonmembers in Training, \$10.00 (E) _____ Medical Technologists, \$40.00 (F) _____ Spouse, \$10.00 (G) _____

Registration includes one copy of the abstract booklet (supplement to *Blood*) and one copy of the Education Program. Additional copies desired _____ (indicate number desired). Supplement to *Blood* \$20.00 _____ Education Program \$6.00 _____

TOTAL ENCLOSED \$ _____
(Payable in US funds only)

Mail this form and check to: ASH Business Office, Registration Supervisor, SLACK, Inc, Box 510, Thorofare, NJ 08086-0510. **Advance registration deadline is November 2, 1987. All registrants who fail to register by November 2, 1987, will be charged an additional fee of \$10.00 upon registration at the Annual Meeting.** (Checks payable to the American Society of Hematology must accompany registration.) Request for refunds must be submitted in writing by December 8, 1987.

*CERTIFICATION FOR NONMEMBERS IN TRAINING

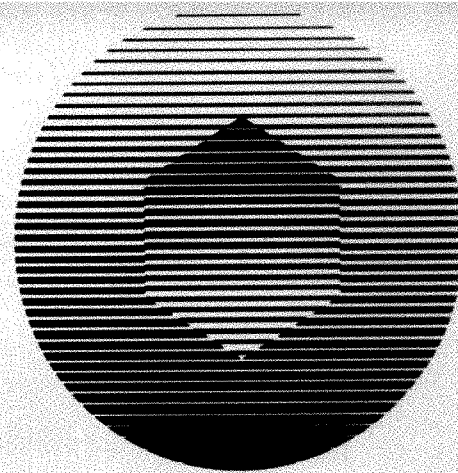
I, _____ certify that Dr _____
(name of training director) (nonmember in training)

is a resident/trainee at _____
(name of institution) (signature of director)

Upon receipt of this Advance Registration, housing forms and additional meeting and social information will be mailed to you.

Grune & Stratton Journals

AMERICAN JOURNAL OF CARDIAC IMAGING	Quarterly
AMERICAN JOURNAL OF KIDNEY DISEASES	Monthly
ARCHIVES OF PSYCHIATRIC NURSING	Bimonthly
BLOOD	Monthly
COMPREHENSIVE PSYCHIATRY	Bimonthly
INFORMATICS IN PATHOLOGY	Quarterly
JOURNAL OF CARDIOTHORACIC ANESTHESIA	Bimonthly
JOURNAL OF CLINICAL ONCOLOGY	Monthly
JOURNAL OF CRITICAL CARE	Quarterly
JOURNAL OF PEDIATRIC NURSING	Bimonthly
JOURNAL OF PEDIATRIC SURGERY	Monthly
JOURNAL OF POST ANESTHESIA NURSING	Quarterly
METABOLISM—CLINICAL AND EXPERIMENTAL	Monthly
PROGRESS IN CARDIOVASCULAR DISEASES	Bimonthly
SEMINARS IN ANESTHESIA	Quarterly
SEMINARS IN ARTHRITIS AND RHEUMATISM	Quarterly
SEMINARS IN DERMATOLOGY	Quarterly
SEMINARS IN DIAGNOSTIC PATHOLOGY	Quarterly
SEMINARS IN HEMATOLOGY	Quarterly
SEMINARS IN NEPHROLOGY	Quarterly
SEMINARS IN NUCLEAR MEDICINE	Quarterly
SEMINARS IN ONCOLOGY	Quarterly
SEMINARS IN ONCOLOGY NURSING	Quarterly
SEMINARS IN OPHTHALMOLOGY	Quarterly
SEMINARS IN ORTHOPAEDICS	Quarterly
SEMINARS IN PERINATOLOGY	Quarterly
SEMINARS IN RESPIRATORY INFECTIONS	Quarterly
SEMINARS IN ROENTGENOLOGY	Quarterly
SEMINARS IN ULTRASOUND, CT, AND MR	Quarterly
SEMINARS IN UROLOGY	Quarterly
SEMINARS IN VETERINARY MEDICINE AND SURGERY (SMALL ANIMAL)	Quarterly
TRANSFUSION MEDICINE REVIEWS	Quarterly (3 issues 1987)
TRANSPLANTATION PROCEEDINGS	Bimonthly



Announcing

RETROVIR[®] (zidovudine)

The first effective treatment for certain
AIDS and other serious HIV* infections

AZT[™] (azidothymidine)
IS NOW

RETROVIR[®] (zidovudine)

*Human immunodeficiency virus

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (zidovudine)

New hope for many AIDS/ARC patients

A multicenter study proved...

RETROVIR can prolong the lives of certain AIDS and ARC patients

- In a double-blind placebo-controlled clinical trial (N = 281), only one death occurred among 144 RETROVIR patients *v* 19 deaths of the 137 patients on placebo ($p < 0.001$). The study population consisted of AIDS patients within 120 days of their first diagnosis of *Pneumocystis carinii* pneumonia or patients with more severe manifestations of AIDS-related complex (ARC)
- The probability of 24-week survival for any patient in the RETROVIR group was 0.98 compared to 0.78 for the placebo group

Comparative Mortality of RETROVIR and Placebo Group Patients in Controlled Clinical Trial (stratified by diagnosis and T4 count) n = 281

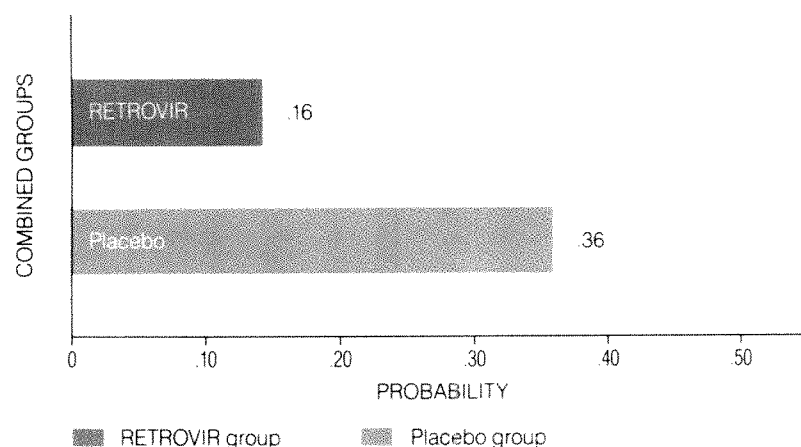
Stratum	Group Assigned	Number of Deaths	Stratum	Group Assigned	Number of Deaths
AIDS (n=160)	RETROVIR	1	Higher T4 count ($\geq 100 \leq 500$) (n=100)	RETROVIR	0
	PLACEBO	12		PLACEBO	4
ARC (n=121)	RETROVIR	0	Lower T4 count (< 100) (n=181)	RETROVIR	1
	PLACEBO	7		PLACEBO	15
Total Deaths			RETROVIR		
			1		
			PLACEBO		
			19		

RETROVIR® (zidovudine) reduced the risk and severity of opportunistic infections

- RETROVIR significantly reduced the probability of acquiring opportunistic infections in AIDS and ARC patients ($p < 0.001$)
- Severity of opportunistic infections was significantly reduced in RETROVIR patients as compared with controls ($p < 0.005$)
- In the controlled trial, no opportunistic infections occurred in ARC patients who had received at least six weeks of RETROVIR therapy. Some ARC patients developed opportunistic infections in the follow-up period while on drug

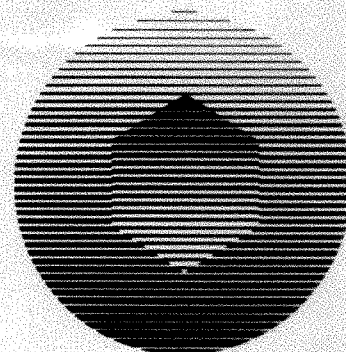
Probability of Developing Opportunistic Infection Within 24 Weeks

(Excludes opportunistic infections acquired within six weeks of entry into the study)



WARNING

WARNING: Therapy with RETROVIR® (zidovudine) is often associated with hematologic toxicity including granulocytopenia and severe anemia requiring transfusions (see WARNINGS). In addition, patients treated with zidovudine may continue to develop opportunistic infections (OIs) and other complications of the acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) caused by the human immunodeficiency virus (HIV). Therefore, patients on zidovudine should be under close clinical observation by physicians experienced in the treatment of patients with diseases associated with HIV. The safety and efficacy of zidovudine have been established only for certain adult AIDS and advanced ARC patients (see INDICATIONS AND USAGE).



IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH



BURROUGHS WELLCOME CO.

Please see brief summary of prescribing information on last page of this advertisement.

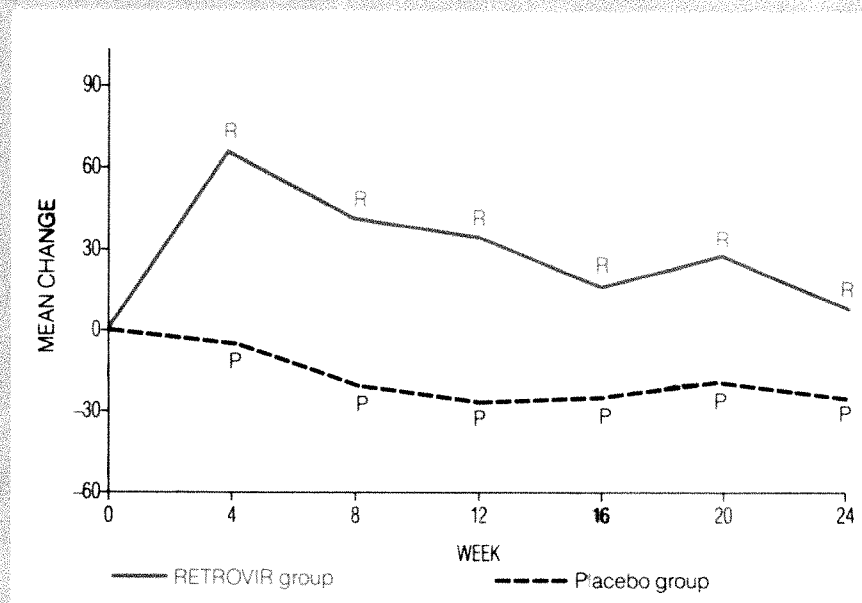
RETROVIR® (zidovudine)

New hope for many AIDS/ARC patients

RETROVIR improved immune status

- T4 cell counts in the RETROVIR group remained significantly higher than in the control group throughout the study
 - In some RETROVIR patients, particularly those with lower T4 cell counts at entry, T4 cell numbers increased, then subsequently declined
 - In contrast, placebo-group patients experienced a progressive decline from baseline in number of T4 cells
- The number of positive delayed hypersensitivity skin test conversions was significantly higher in the RETROVIR group ($p < 0.001$)

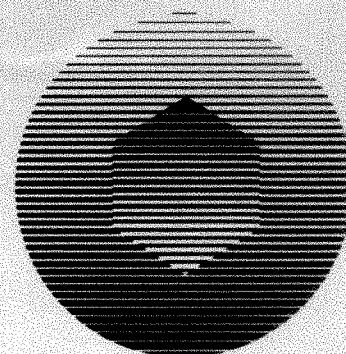
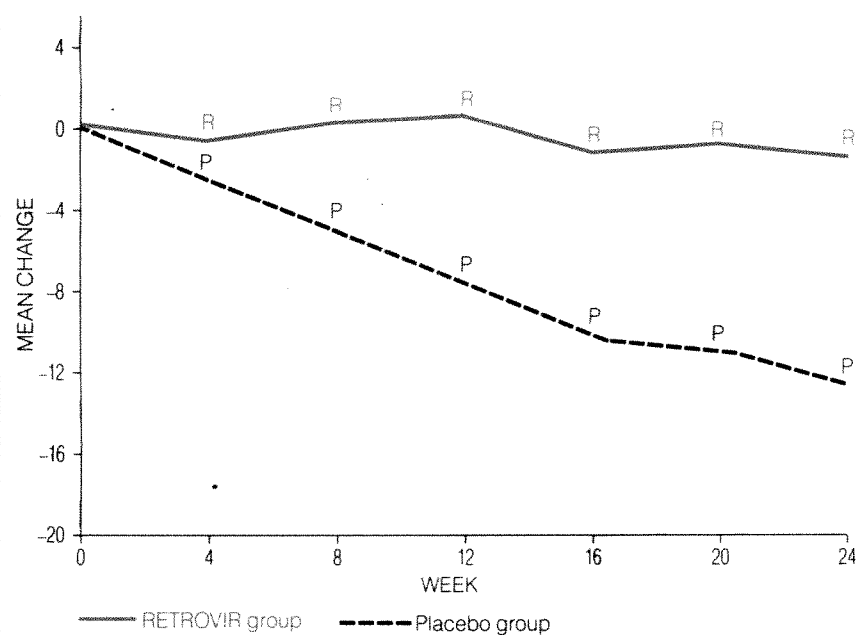
Change in T4 Cell Count



Patients' general health improved

- Patients on RETROVIR® (zidovudine) maintained their high Karnofsky performance scores (an index measuring ability to perform activities of daily life), whereas placebo group patients' scores declined
- RETROVIR patients experienced significantly fewer symptoms related to HIV infection than those on placebo
- Patients on RETROVIR therapy maintained their body weight throughout the study, whereas placebo group patients progressively lost weight

Change in Karnofsky Score for All AIDS/ARC Patients Combined



IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH



BURROUGHS WELLCOME CO.

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RETROVIR® (zidovudine)

New hope for many AIDS/ARC patients

Clinical studies showed...

It is important to identify patients at greatest risk for bone marrow toxicity, and subsequent RBC and WBC suppression, and monitor carefully

- 25% of RETROVIR patients developed anemia (<7.5 g/dl) as compared to 4% in the placebo group. This adverse reaction was most likely to occur in AIDS patients and patients with lower numbers of T4 cells
- 31% of RETROVIR patients required at least one transfusion, compared with 11% of placebo patients
- Granulocyte depression ($<750/\text{mm}^3$) was observed in 39% of RETROVIR patients as compared with 9% of placebo patients. Granulocytopenia occurred more frequently in AIDS patients and patients with lower T4 cell counts

Clinical adverse events

- Although the majority of patients in *both* the RETROVIR (84%) and control groups (72%) reported the occurrence of at least one adverse event, nausea, myalgia, insomnia, and moderate-to-severe headache were significantly more frequent among RETROVIR patients

The potential hematologic toxicity of RETROVIR plays an important role in patient management

- The majority of patients who required adjustment of RETROVIR dosage had hematologic toxicity, specifically anemia or granulocytopenia, usually occurring within the first four to eight weeks of RETROVIR therapy
- Because of this potential, careful monitoring of hematologic indices is recommended every two weeks*

Drug interactions must be considered with RETROVIR

- Concomitant use of acetaminophen with RETROVIR may potentiate granulocytopenia; other drugs that may interfere with RETROVIR metabolism or excretion (eg, probenecid, lorazepam, indomethacin, cimetidine) should be avoided



RETROVIR—the newest antiviral breakthrough from Burroughs Wellcome Co.—is the first effective treatment for certain HIV infections.

*RETROVIR dose interruption may be required for significant anemia or granulocytopenia until evidence of bone marrow recovery is observed. A reduction in daily dose may be adequate for less severe depression of RBC/WBC counts.

**Since the initial supply of RETROVIR®
(zidovudine) is limited, a special distribution
program is in place**

- Rapidity of development and complexity of the production process have limited the current supply of RETROVIR
- To help ensure a continuous supply of RETROVIR for patients at greatest need and those who would benefit most from treatment during this period of limited supply, a special distribution program has been established
- An independent group of experts from the Infectious Disease Society of America (IDSA) has assisted in developing this program for the best utilization of RETROVIR

**Patients can be enrolled in the RETROVIR
distribution program now**

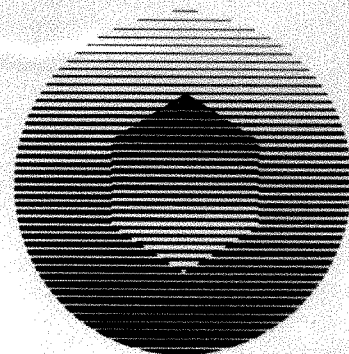
- You can apply to enroll your patients in the RETROVIR distribution program at this time
- If you are currently treating AIDS or ARC patients and need additional information, call 1-800-843-9388
- Detailed information and enrollment applications are also available from your Burroughs Wellcome Co. Sales Representative
- Only patients enrolled in the distribution program will be able to receive RETROVIR

**Burroughs Wellcome Co.:
Leader in Antiviral Research**

- Continuing clinical trials to evaluate further the role of RETROVIR in the treatment of HIV infections
- Conducting research for antiviral agents effective against varicella-zoster, Epstein-Barr, human papilloma, hepatitis B virus, and cytomegalovirus
- Providing physicians with antiviral products effective against herpes simplex — today's hope for patients with genital herpes
- Pledging to keep you updated as new information becomes available

*If you have patients with
HIV infections and need
additional information,
please call*

1-800-843-9388



IMPROVING LIVES THROUGH
ANTIVIRAL RESEARCH



BURROUGHS WELLCOME CO.

Please see brief summary of prescribing information on last page of this advertisement.

RETROVIR® (ZIDOVUDINE) Capsules

WARNING: THERAPY WITH RETROVIR (ZIDOVUDINE) IS OFTEN ASSOCIATED WITH HEMATOLOGIC TOXICITY INCLUDING GRANULOCYTOPENIA AND SEVERE ANEMIA REQUIRING TRANSFUSIONS (SEE WARNINGS).

IN ADDITION, PATIENTS TREATED WITH ZIDOVUDINE MAY CONTINUE TO DEVELOP OPPORTUNISTIC INFECTIONS (OIs) AND OTHER COMPLICATIONS OF THE ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) AND AIDS RELATED COMPLEX (ARC) CAUSED BY THE HUMAN IMMUNODEFICIENCY VIRUS (HIV). THEREFORE, PATIENTS ON ZIDOVUDINE SHOULD BE UNDER CLOSE CLINICAL OBSERVATION BY PHYSICIANS EXPERIENCED IN THE TREATMENT OF PATIENTS WITH DISEASES ASSOCIATED WITH HIV. THE SAFETY AND EFFICACY OF ZIDOVUDINE HAVE BEEN ESTABLISHED ONLY FOR CERTAIN ADULT AIDS AND ADVANCED ARC PATIENTS (SEE "INDICATIONS AND USAGE").

INDICATIONS AND USAGE: Retrovir Capsules are indicated for the management of certain adult patients with symptomatic HIV infection (AIDS and advanced ARC) who have a history of cytologically confirmed *Pneumocystis carinii* pneumonia (PCP) or an absolute CD4 (T4 helper/inducer) lymphocyte count of less than 200/mm³ in the peripheral blood before therapy is begun.

This indication is based primarily on the results of a randomized, double-blind, placebo-controlled trial conducted at 12 medical centers in the United States in which 281 patients with AIDS or advanced ARC were studied for an average of four and a half months. Additional data have been collected on approximately 80% of these patients who have received zidovudine in an open-label extension of this trial for an average of five more months.

The patient population of the controlled trial consisted of 150 AIDS patients (85 Retrovir and 75 placebo) who had recovered from their first episode of PCP diagnosed within the previous four months, and 121 ARC patients (59 Retrovir and 62 placebo) with multiple signs and symptoms of HIV infection, including mucocutaneous candidiasis and/or unexplained weight loss (≥ 15 lbs or $> 10\%$ of prior body weight). All patients had evidence of impaired cellular immunity with an absence of delayed cutaneous hypersensitivity and a decreased number of CD4 (T4) lymphocytes in the peripheral circulation. Two hundred twenty-one (79% of all patients) had fewer than 200 T4 cells/mm³ at entry (95% of AIDS patients and 57% of ARC patients). All patients began therapy at a dose of 250 mg every 4 hours around the clock. This dosage was reduced or temporarily or permanently discontinued if serious marrow toxicity occurred. The trial was stopped because of a significant reduction in mortality before all patients had completed the planned 24 weeks of treatment. There were 19 deaths in the placebo group and 1 in the Retrovir group ($p < .001$). All deaths were apparently due to opportunistic infections (OI) or other complications of HIV infection. Treatment duration ranged from 12 weeks to 26 weeks, with a mean and median duration of 17 and 18 weeks, respectively.

Retrovir also significantly reduced the risk of acquiring an AIDS-defining OI in patients after 6 weeks of treatment ($p < .001$). In addition, patients who received Retrovir generally did better than the placebo group in terms of several other measures of efficacy including performance level, neuropsychiatric function, maintenance of body weight and the number and severity of symptoms associated with HIV infection. A modest increase in mean CD4 (T4) counts was seen in the zidovudine group but the significance of this finding is unclear as the CD4 (T4) counts declined again in some patients.

The most significant adverse reaction noted in the study was a depression of formed elements in the peripheral blood, which necessitated dose reduction or drug discontinuation in 49 of the 144 (34%) patients receiving Retrovir. Of those participants whose baseline CD4 (T4) lymphocyte counts were less than 200, 47% of those receiving Retrovir and 10% of those receiving placebo developed a granulocyte count of $< 750/\text{mm}^3$. Similarly, 45% of Retrovir recipients and only 14% of placebo recipients had a 25% or greater reduction in hemoglobin. (See ADVERSE REACTIONS.)

At the conclusion of the placebo-controlled trial, 127 Retrovir recipients and 100 placebo recipients elected to enroll in an uncontrolled extension protocol in which all patients received Retrovir at a dose of 200 mg every four hours. This dose was chosen because of concern about cumulative hematologic toxicity and to allow for greater flexibility in dosing. Over the subsequent five months, opportunistic infections continued to occur and additional patients died in both groups. Sixteen patients from the original placebo cohort died after the conclusion of the controlled trial. Four of these 16 never received Retrovir and seven expired during the first month of therapy. Ten additional deaths occurred among the original Retrovir recipients. With respect to these data, termination of the controlled trial precludes direct comparisons of mortality and morbidity between the original placebo and drug recipients. Long-term follow-up of these patients, however, will allow for examination of the natural history of prolonged zidovudine therapy.

CONTRAINDICATIONS: Retrovir Capsules are contraindicated for patients who have potentially life-threatening allergic reactions to any of the components of the formulation.

WARNINGS: Zidovudine has been carefully studied in limited numbers of seriously ill HIV-infected patients treated for a limited period of time. Therefore, the full safety and efficacy profile of zidovudine has not been completely defined, particularly in regard to prolonged use, and especially in HIV-infected individuals who have less advanced disease.

Zidovudine should be used with extreme caution in patients who have bone marrow compromise evidenced by granulocyte count $< 1000/\text{mm}^3$ or hemoglobin < 9.5 g/dl. In the placebo-controlled study, anemia and granulocytopenia were the most significant adverse events observed (see ADVERSE REACTIONS).

Significant anemia most commonly occurred after 4 to 6 weeks of therapy and in many cases required dose adjustment, discontinuation of zidovudine, and/or blood transfusions. Frequent (at least every 2 weeks) blood counts are strongly recommended in patients taking zidovudine. If anemia or granulocytopenia develops, dosage adjustments may be necessary (see DOSAGE AND ADMINISTRATION).

Coadministration of zidovudine with other drugs metabolized by glucuronidation should be avoided because the toxicity of either drug may be potentiated (see Drug Interactions under PRECAUTIONS). Zidovudine recipients who used acetaminophen during the controlled trial had an increased incidence of granulocytopenia which appeared to be correlated with the duration of acetaminophen use.

PRECAUTIONS:

General: Zidovudine is eliminated from the body primarily by renal excretion following metabolism in the liver (glucuronidation). There are currently no data available concerning the use of zidovudine in patients with impaired renal or hepatic function, and such patients may be at a greater risk of toxicity from zidovudine.

Drug Interactions: The interaction of other drugs with zidovudine has not been studied in a systematic manner. Coadministration of zidovudine with drugs that are nephrotoxic, cytotoxic, or which interfere with RBC/WBC number or function (e.g., dapsone, pentamidine, amphotericin B, flucytosine, vincristine, vinblastine, adriamycin, or interferon) may increase the risk of toxicity. Limited data suggest that probenecid may inhibit glucuronidation and/or reduce renal excretion of zidovudine. In addition, other drugs (e.g., acetaminophen, aspirin, or indomethacin) may competitively inhibit glucuronidation (see WARNINGS).

Some experimental nucleoside analogues which are being evaluated in AIDS and ARC patients may affect RBC/WBC number or function and may increase the potential for hematologic toxicity of zidovudine. Some experimental nucleoside analogues affecting DNA replication antagonize the *in vitro* antiviral activity of zidovudine against HIV and thus, concomitant use of such drugs should be avoided.

Some drugs such as trimethoprim-sulfamethoxazole, pyrimethamine, and acyclovir may be necessary for the management or prevention of opportunistic infections. In the controlled trial, increased toxicity was not detected with limited exposure to these drugs. However, there are two published reports of neurotoxicity (one of profound lethargy and one of seizure) associated with concomitant use of zidovudine and acyclovir.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Long-term carcinogenicity studies of zidovudine in animals have not been completed. However, in an *in vitro* mammalian cell transformation assay, zidovudine was positive at concentrations of 0.5 $\mu\text{g}/\text{ml}$ and higher.

No evidence of mutagenicity (with or without metabolic activation) was observed in the Ames *Salmonella* mutagenicity assay. In a mutagenicity assay conducted in L5178Y/TK⁺ mouse lymphoma cells, zidovudine was weakly mutagenic in the absence of metabolic activation only at the highest concentrations tested (4000 and 5000 $\mu\text{g}/\text{ml}$). In the presence of metabolic activation, the drug was weakly mutagenic at concentrations of 1000 $\mu\text{g}/\text{ml}$ and higher. In an *in vitro* cytogenetic study performed in cultured human lymphocytes, zidovudine induced dose-related structural chromosomal abnormalities at concentrations of 3 $\mu\text{g}/\text{ml}$ and higher. No such effects were noted at the two lowest concentrations tested, 0.3 and 1 $\mu\text{g}/\text{ml}$.

Pregnancy: Pregnancy Category C. An oral teratology study in pregnant rats using doses up to 20 times the human dose has revealed no evidence of harm to the fetus due to zidovudine. It is not known whether

zidovudine can cause fetal harm when administered to a pregnant woman or can affect reproductive capacity. Zidovudine should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether zidovudine is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions from zidovudine in nursing infants, mothers should be instructed to discontinue nursing if they are receiving zidovudine.

Pediatric Use: Safety and effectiveness in children have not been established.

ADVERSE REACTIONS: The most frequent adverse events and abnormal laboratory values reported in the placebo-controlled clinical trial of oral zidovudine administration in 281 patients (144 patients zidovudine, 137 patients placebo) were granulocytopenia and anemia. The occurrence of these hematologic toxicities was inversely related to CD4 (T4) lymphocyte number, hemoglobin, and granulocyte count at study entry, and directly related to dose and duration of therapy. The frequency of granulocytopenia and anemia according to the patients' CD4 (T4) levels is shown in the following table:

Abnormality	Pretreatment CD4 (T4) Levels			
	$\leq 200/\text{mm}^3$		$> 200/\text{mm}^3$	
	Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia ($< 750/\text{mm}^3$)	47%	10%	10%	3%
Anemia (Hgb < 7.5 g/dl)	30%	6%	3%	0%

Because many patients were anemic and/or granulocytopenic before starting therapy with zidovudine, examining the degree of change when compared to baseline, as shown in the table below, may be more informative.

Abnormality	% Decrease from Baseline	Pretreatment CD4 (T4) Levels			
		$\leq 200/\text{mm}^3$		$> 200/\text{mm}^3$	
		Zidovudine (n = 113)	Placebo (n = 105)	Zidovudine (n = 30)	Placebo (n = 30)
Granulocytopenia	$> 50\%$	55%	19%	40%	13%
Anemia	$> 25\%$	45%	14%	10%	10%

The anemia appeared to be the result of impaired erythrocyte maturation as evidenced by increasing macrocytosis (MCV) while on drug.

The 281 patients treated in this controlled trial had serious underlying disease with multiple baseline symptoms and clinical abnormalities. The following table summarizes those reported clinical adverse events which occurred in at least 5% of all patients treated with zidovudine. Severe headache, nausea, insomnia and myalgia were reported at a significantly greater rate in zidovudine recipients.

Percentage (%) of Patients with Clinical Events					
Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %	Adverse Event	Zidovudine (n = 144) %	Placebo (n = 137) %
BODY AS A WHOLE			MUSCULOSKELETAL		
Asthenia	19	18	Myalgia	8	2
Diaphoresis	5	4			
Fever	16	12	NERVOUS		
Headache	42	37	Dizziness	6	4
Malaise	8	7	Insomnia	5	1
			Paresthesia	6	3
GASTROINTESTINAL			Somnolence	8	9
Anorexia	11	8	RESPIRATORY		
Diarrhea	12	18	Dyspnea	5	3
Dyspepsia	5	4			
GI Pain	20	19	SKIN		
Nausea	46	18	Rash	17	15
Vomiting	6	3	SPECIAL SENSES		
			Taste Perversion	5	8

Clinical adverse events which occurred in less than 5% of all patients treated with zidovudine are listed below. Since many of these adverse events were seen in placebo-treated patients as well as zidovudine recipients, they may not be attributable to the drug.

Body as a whole: body odor, chills, edema of the lip, flu syndrome, hyperalgesia, back pain, chest pain, lymphadenopathy.

Cardiovascular: vasodilation.

Gastrointestinal: constipation, dysphagia, edema of the tongue, eructation, flatulence, bleeding gums, rectal hemorrhage, mouth ulcer.

Musculoskeletal: arthralgia, muscle spasm, tremor, twitch.

Nervous: anxiety, confusion, depression, emotional lability, nervousness, syncope, loss of mental acuity, vertigo.

Respiratory: cough, epistaxis, pharyngitis, rhinitis, sinusitis, hoarseness.

Skin: acne, pruritus, urticaria.

Special senses: amblyopia, hearing loss, photophobia.

Urogenital: dysuria, polyuria, urinary frequency, urinary hesitancy.

DOSAGE AND ADMINISTRATION: The recommended starting dose of Retrovir (zidovudine) is 200 mg (two 100 mg capsules) administered orally every four hours around the clock.

Hematologic toxicities appear to be related to pretreatment bone marrow reserve and to dose and duration of therapy. Careful monitoring of hematologic indices every two weeks is recommended to detect serious anemia or granulocytopenia. In patients with hematologic toxicity, reduction in hemoglobin may occur as early as 2 to 4 weeks, and granulocytopenia usually occurs after 6 to 8 weeks.

Dose Adjustment: Significant anemia (hemoglobin of < 7.5 g/dl or reduction of $> 25\%$ of baseline) and/or significant granulocytopenia (granulocyte count of $< 750/\text{mm}^3$ or reduction of $> 50\%$ from baseline) may require a dose interruption until some evidence of marrow recovery is observed. For less severe anemia or granulocytopenia, a reduction in daily dose may be adequate. In patients who develop significant anemia, dose modification does not necessarily eliminate the need for transfusion. If marrow recovery occurs following dose modification, gradual increases in dose may be appropriate depending on hematologic indices and patient tolerance.



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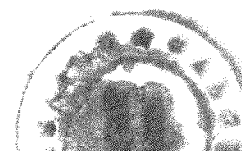
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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. *Br J Haematol* 38:161, 1978

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3. Lillie RD: *Histopathologic Technic and Practical Histochemistry* (ed 3). New York, Blakiston, 1965, p 39

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6. Poliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

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7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. *Clin Res* 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. *Blood* 47:879, 1976 (letter)

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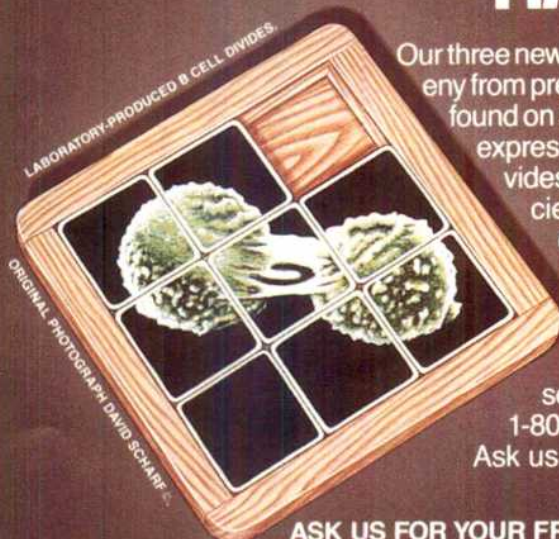
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Immune Globulin Intravenous (Human) GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8 ± 0.4 .

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average, the distribution of IgG subclasses present in this product is the same as is present in normal plasma.¹ Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from plasma units found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum levels is, therefore, to be expected.²

As a class, IgG survives longer *in vivo* than other serum proteins.^{2,3} Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.^{2,3,4} The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.^{2,3,4,5}

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has

been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.^{4,5}

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 µg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials, such reactions have been observed with other immunoglobulin products.^{5,6} Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea may occasionally occur. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials, are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS.)

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by

monitoring clinical response and/or the serum IgG levels before each dose, to insure that they do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/kg per Hr be used. If infusion at this rate causes the patient no distress, the administration rate may be gradually increased but should not exceed 4 mL/kg per Hr.

A rate of administration which is too rapid may cause flushing and changes in pulse rate and blood pressure. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Human), GAMMAGARD should be administered as soon after reconstitution as possible. Administration should not begin more than 2 hours after reconstitution.

The reconstituted material should be at room temperature during administration.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

Follow directions for use which accompany the administration set. If a Hyland set is not used, make sure the administration set contains an adequate filter.

How Supplied

Immune Globulin Intravenous (Human), GAMMAGARD is supplied in either 2.5 g or 5.0 g single use vials. Each vial of Immune Globulin Intravenous (Human), GAMMAGARD is furnished with a suitable volume of Sterile Water for Injection, USP, a transfer device and an administration set which contains an integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Human), GAMMAGARD should be stored under ordinary refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing should be avoided to prevent the diluent bottle from breaking.

REFERENCES

1. Unpublished data in the files of Travenol Laboratories, Inc.
2. Waldmann TA, Storer W: Metabolism of immunoglobulins. *Prog Allergy* 13: 1-110, 1969
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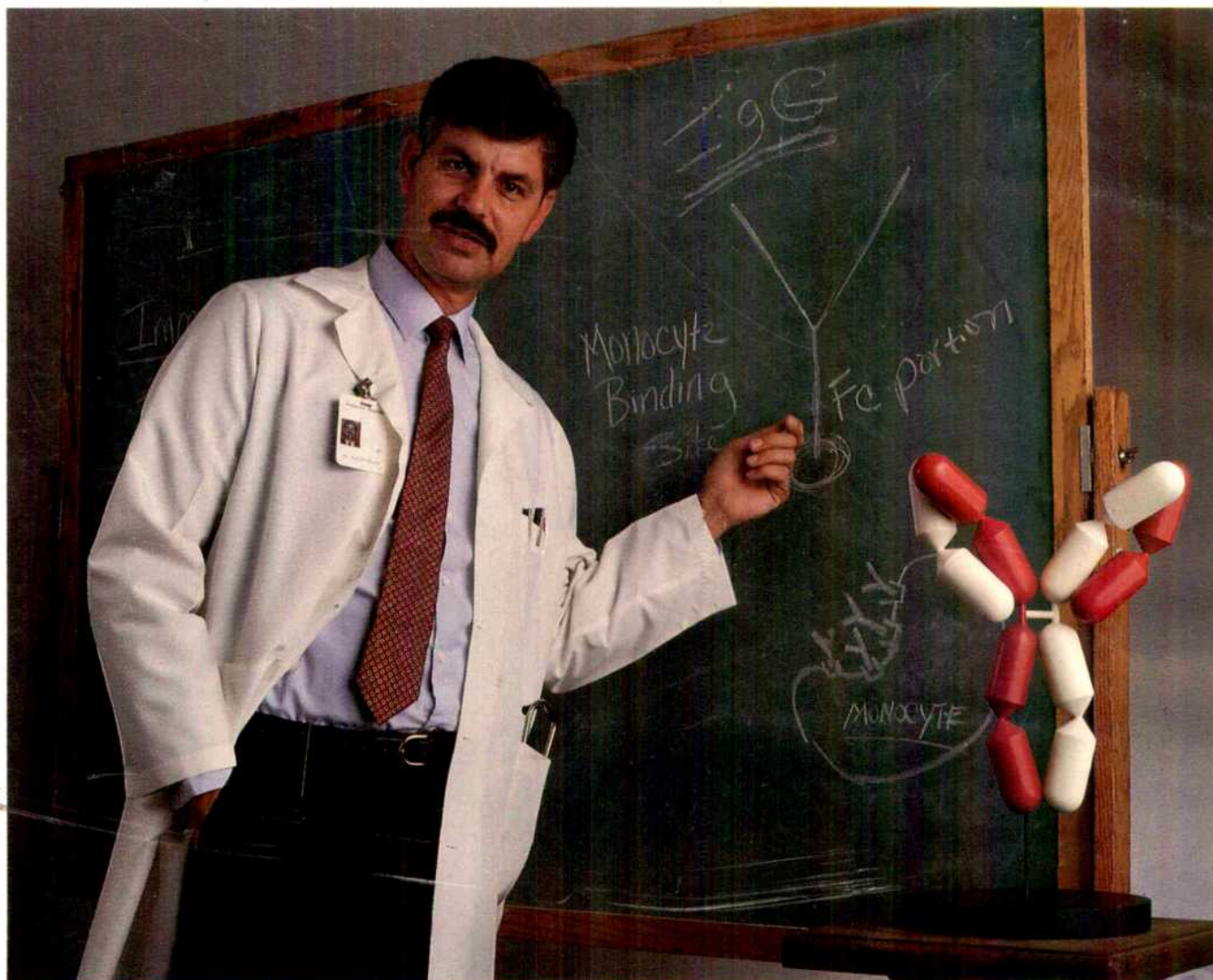


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¹Jungi, TW, Santer, M., Lerch, PG., Barandun, S.: Effect of Various Treatments of Gamma-Globulin (IgG) for Achieving Intravenous Tolerance on the Capacity to Interact with Human Monocyte Fc Receptors. Vox Sang 51:18-26 (1986)

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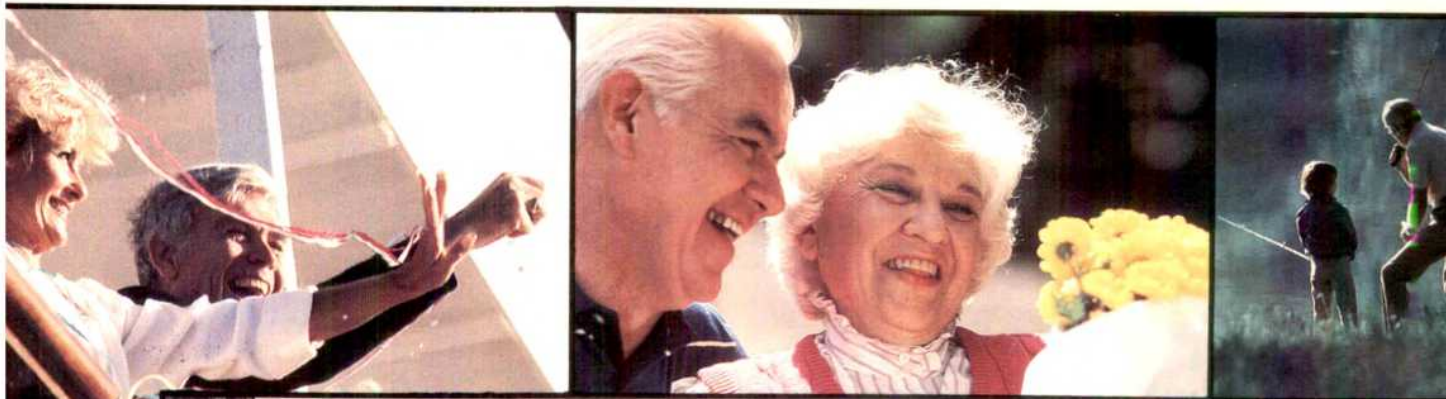
*American Society of Hematology
Twenty-Ninth Annual Meeting
December 5-8, 1987
Washington, DC
(see page 602 for registration information)*

ABSTRACT DEADLINE: SEPTEMBER 1, 1987

Table of Contents v

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INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g., methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below $5 \times 10^{-8} M$.^{1,2}

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e., trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

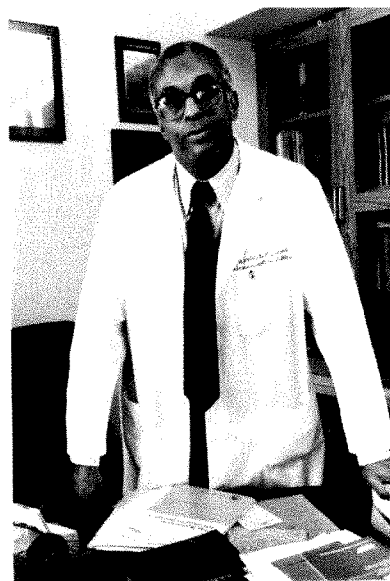
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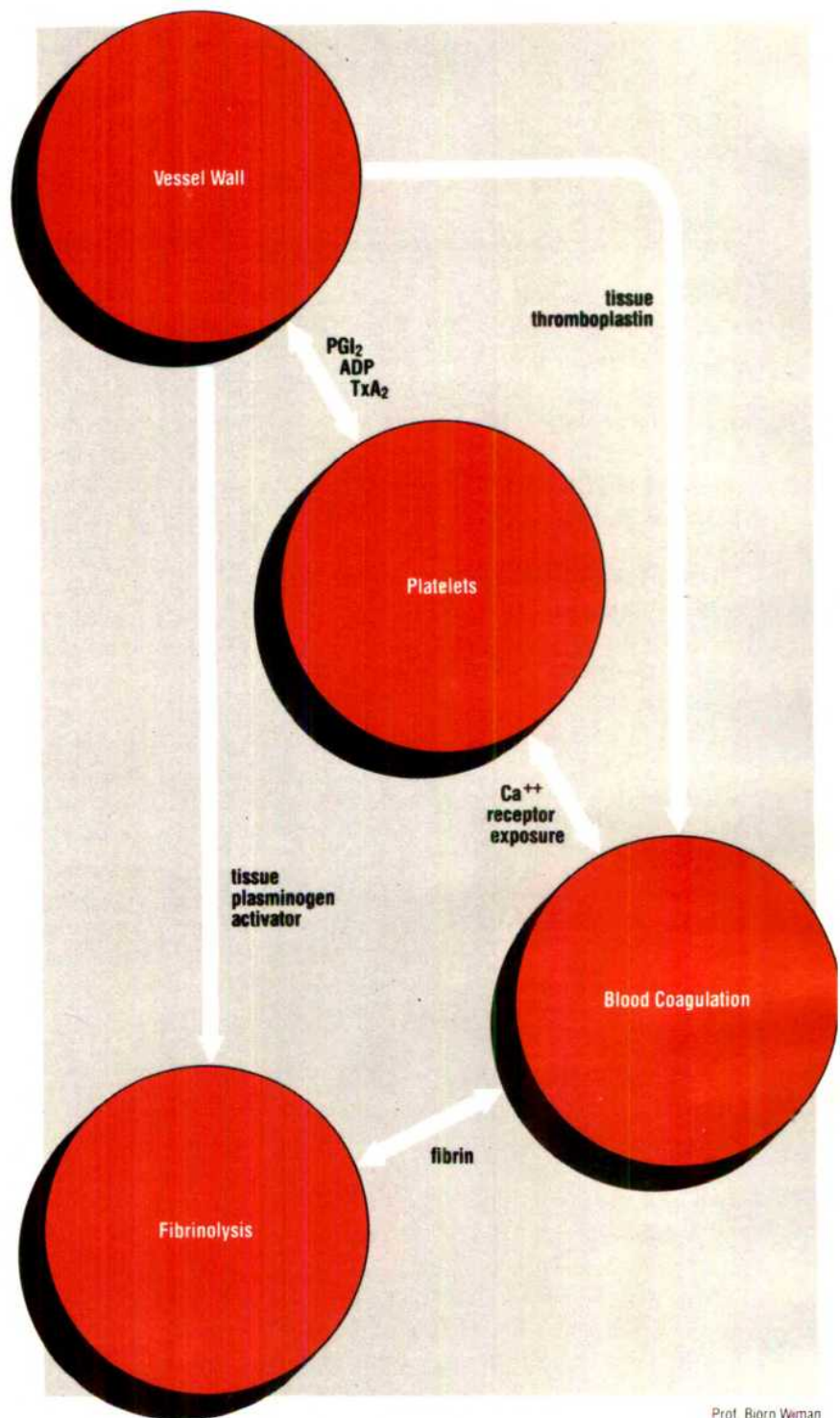
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*The Journal of
The American Society of Hematology*

VOL 70, NO 2

AUGUST 1987

CONTENTS

Review: The Pathophysiology of the Prethrombotic State in Humans: Insights Gained From Studies Using Markers of Hemostatic System Activation.....	<i>Kenneth A. Bauer and Robert D. Rosenberg</i>	343
Characterization of the Chemotactic Defect in Polymorphonuclear Leukocytes Exposed to Influenza Virus In Vitro.....	<i>Dorothy L. Moore and Elaine L. Mills</i>	351
Tyrosine Kinase and Phosphotyrosine Phosphatase Activity in Human Promyelocytic Leukemia Cells and Human Polymorphonuclear Leukocytes	<i>Andrew S. Kraft and Roger L. Berkow</i>	356
Actin Polymerization and Its Relationship to Locomotion and Chemokinetic Response in Maturing Human Promyelocytic Leukemia Cells.....	<i>William H. Meyer and Thomas H. Howard</i>	363
The c-sis Gene Expression in Cells From a Patient With Acute Megakaryoblastic Leukemia and Down's Syndrome.....	<i>Shousuke Sunami, Akira Fuse, Bunsiti Simizu, Mitsuoki Eguchi, Yasuhide Hayashi, Kanji Sugita, Shinpei Nakazawa, Yuri Okimoto, Takeyuki Sato, and Hironori Nakajima</i>	368
Characterization of a New Human Diploid Myeloid Leukemia Cell Line (PLB-985) With Granulocytic and Monocytic Differentiating Capacity	<i>Kent A. Tucker, Michael B. Lilly, Louis Heck, Jr, and Thomas A. Rado</i>	372
Eosinophils Do Respond to fMLP.....	<i>Maria Yazdanbakhsh, Carel M. Eckmann, Leo Koenderman, Arthur J. Verhoeven, and Dirk Roos</i>	379
Productive Infection by B19 Parvovirus of Human Erythroid Bone Marrow Cells In Vitro	<i>Keiya Ozawa, Gary Kurtzman, and Neal Young</i>	384
Homosexual Men With Thrombocytopenia Have Impaired Reticuloendothelial System Fc Receptor-Specific Clearance.....	<i>Bradley S. Bender, Thomas C. Quinn, and Jerry L. Spivak</i>	392
High Serum Levels of Soluble Interleukin 2 Receptor in Patients With B Chronic Lymphocytic Leukemia.....	<i>G. Semenzato, R. Foa, C. Agostini, R. Zambello, L. Trentin, F. Vinante, F. Benedetti, M. Chilosi, and G. Pizzolo</i>	396
The Effects of Purified Recombinant Murine Interleukin-3 and/or Purified Natural Murine CSF-1 In Vivo on the Proliferation of Murine High- and Low-Proliferative Potential Colony-Forming Cells: Demonstration of In Vivo Synergism	<i>Douglas E. Williams, Gao Hangoc, Scott Cooper, H. Scott Boswell, Richard K. Shadduck, Steven Gillis, Abdul Waheed, David Urdal, and Hal E. Broxmeyer</i>	401
Recombinant Human Granulocyte Colony-Stimulating Factor Repairs the Abnormalities of Neutrophils in Patients With Myelodysplastic Syndromes and Chronic Myelogenous Leukemia	<i>Akiro Yuo, Seiichi Kitagawa, Tetsuro Okabe, Akio Urabe, Yoshinori Komatsu, Seiga Itoh, and Fumimaro Takaku</i>	404
Hypermethylation of the 5' Region of the Calcitonin Gene Is a Property of Human Lymphoid and Acute Myeloid Malignancies.....	<i>Stephen B. Baylin, Eric R. Fearon, Bert Vogelstein, Andr��e de Bustros, Saul J. Sharkis, Philip J. Burke, Stephen P. Staal, and Barry D. Nelkin</i>	412
Normal Cellular Counterparts of B Cell Chronic Lymphocytic Leukemia.....	<i>Arnold S. Freedman, Andrew W. Boyd, Frederick R. Bieber, John Daley, Karen Rosen, Jack C. Horowitz, David N. Levy, and Lee M. Nadler</i>	418
Pseudo-Bernard-Soulier Syndrome: Thrombocytopenia Caused by Autoantibody to Platelet Glycoprotein Ib.....	<i>Dana V. Devine, Mark S. Currie, Wendell F. Rosse, and Charles S. Greenberg</i>	428
A Colony Assay for In Vitro Transformation by Human T Cell Leukemia Viruses Type I and Type II.....	<i>Mordechai Aboud, David W. Golde, Noelle Bersch, Joseph D. Rosenblatt, and Irvin S.Y. Chen</i>	432

(Continued on following page)

(Contents continued)

Monoclonal Antibodies Against the Chronic Lymphatic Leukemia Antigen cLLa: Characterization and Reactivity <i>Guy B. Faguet and Julia F. Agee</i>	437
Intermediate- to High-Grade Histology of Lymphomas Carrying t(14;18) Is Associated With Additional Nonrandom Chromosome Changes..... <i>Mark E. Richardson, Chen Quanguang, Daniel A. Filippa, Kemeth Offit, Alice Hampton, Prasad R.K. Koduru, Suresh C. Jhanwar, Philip H. Lieberman, Bayard D. Clarkson, and R.S.K. Chaganti</i>	444
CML Patients in Blast Crisis Have Breakpoints Localized to a Specific Region of the BCR..... <i>Kim Schaefer-Rego, Henryk Dudek, Daniel Popenoe, Zalmen Arlin, J. Gregory Mears, Arthur Bank, and David Leibowitz</i>	448
The Protooncogene <i>c-fos</i> Is Transcriptionally Active in Normal Human Granulocytes <i>K. Heidorn, H. Kreipe, H.J. Radzun, R. Müller, and M.R. Parwaresch</i>	456
Age-Related Deficiency of the Synthesis of Platelet Activating Factor by Leukocytes From Zellweger Patients <i>A. Sturk, M.C.L. Schaap, A. Prins, J.W. ten Cate, L.C.P. Govaerts, R.J.A. Wanders, H.S.A. Heymans, and R.B.H. Schutgens</i>	460
Simian Virus 40-Transformed Adherent Cells From Human Long-term Marrow Cultures: Cloned Cell Lines Produce Cells With Stromal and Hematopoietic Characteristics <i>Jack W. Singer, Pierre Charbord, Armand Keating, John Nemunaitis, Greg Raugi, Thomas N. Wight, Jose A. Lopez, Gerald J. Roth, Lois W. Dow, and Philip J. Fialkow</i>	464
Increased Surface Expression of the Membrane Glycoprotein IIb/IIIa Complex Induced by Platelet Activation. Relationship to the Binding of Fibrinogen and Platelet Aggregation..... <i>Kenji Niiya, Elisabeth Hodson, Rosella Bader, Vicky Byers-Ward, James A. Koziol, Edward F. Plow, and Zaverio M. Ruggeri</i>	475
Photoradiation Models for the Clinical Ex Vivo Treatment of Autologous Bone Marrow Grafts..... <i>Jens Atzpödien, Subhash C. Gulati, Annabel Strife, and Bayard D. Clarkson</i>	484
An Inhibitory Monoclonal Antibody Against Human Tissue Factor <i>Steven D. Carson, Sherman E. Ross, Ronald Bach, and Arabinda Guha</i>	490
Lymphocyte Interleukin 2 Production and Responsiveness Are Altered in Patients With the Primary Myelodysplastic Syndrome..... <i>Olca Aynlar-Batuman, Jerry Shevitz, Ursula C. Traub, Scott Murphy, and Daniel Szejewski</i>	494
Isolation, Characterization, and Localization of Glycosaminoglycans in Rabbit Bone Marrow <i>Kayoko Oguri, Eiko Okayama, Bruce Caterson, and Minoru Okayama</i>	501
ADP Causes Subsecond Changes in Protein Phosphorylation of Platelets <i>D.J. Carty, D.L. Freas, and A.R.L. Gear</i>	511
Effects of Herpes Virus Carrier Status on Peripheral T Lymphocyte Subsets <i>Jan W. Gratama, Albert M.I.H. Naipal, Maria A.P. Oosterveer, Theo Stijnen, Hanneke C. Kluin-Nelemans, Leo A. Ginsel, Gerard J. den Ottolander, Anton C. Hekker, Joe D'Amato, Marijke van der Giessen, and Hans J. Tanke</i>	516
Capacity of Human Serum to Depolymerize Actin Filaments <i>Paul A. Janmey and Stuart E. Lind</i>	524
Hemophilia A: Carrier Detection and Prenatal Diagnosis by DNA Analysis <i>Mario Pecorara, Lucia Casarino, Pier Giorgio Mori, Massimo Morfini, Giuseppe Mancuso, Angela Maria Scrivano, Elio Boeri, Angelo Claudio Molinari, Raffaello De Biasi, Nicola Ciavarella, Franco Bencivelli, Teo Ripa, Guido Barbujani, Angela Loi, Lucia Perseu, Antonio Cao, and Mario Pirastu</i>	531
Immunoglobulin and T Cell Receptor Gene Configuration in Acute Lymphoblastic Leukemia of Infancy <i>Carolyn A. Felix, Gregory H. Reaman, Stanley J. Korsmeyer, Gregory F. Hollis, Patricia A. Dimdorf, John J. Wright, and Ilan R. Kirsch</i>	536
Erythropoietin Production by the Fetal Liver in an Adult Environment <i>Alan W. Flake, Michael R. Harrison, N. Scott Adzick, and Esmail D. Zanjani</i>	542
Effect of Monoclonal Antibodies Against von Willebrand Factor and Platelet Glycoproteins IIb/IIIa on the Platelet Retention Test <i>Jean McPherson, Sandra Brownlee, and Marjorie B. Zucker</i>	546
Inhibition of Heparanase-Mediated Degradation of Extracellular Matrix Heparan Sulfate by Non-anticoagulant Heparin Species <i>Matia Bar-Ner, Amiram Eldor, Lina Wasserman, Yaacov Matzner, Zvi Fuks, and Israel Vlodavsky</i>	551

(Continued on following page)

(Contents continued)

- ADP-Induced Platelet Aggregation Depends on the Conformation or Availability of the Terminal Gamma Chain Sequence of Fibrinogen. Study of the Reactivity of Fibrinogen Paris 1 *Marie-Hélène Denninger, Martine Jandrot-Perrus, Jacques Elion, Olivier Bertrand, Gene A. Homandberg, Michael W. Mosesson, and Marie-Claude Guillin* 558

CONCISE REPORTS

- Blood Cells Participate in the Fibrinolytic Response to Tissue-Type Plasminogen Activator
M. Wahbi Hammouda and Leonard A. Moroz 564
- Deoxycytidine Preferentially Protects Normal Versus Leukemic Myeloid Progenitor Cells From Cytosine Arabinoside-Mediated Cytotoxicity *Kapil Bhalla, William MacLaughlin, John Cole, Zalmen Arlin, Michael Baker, Gary Graham, and Steven Grant* 568
- Eosinophils Stimulate Fibroblast DNA Synthesis *Stephanie H. Pincus, Kashi S. Ramesh, and David J. Wyler* 572
- Detection of HIV Antigen and Specific Antibodies to HIV Core and Envelope Proteins in Sera of Patients With HIV Infection *Yunzhen Cao, Fred Valentine, Sally Hojvat, Jean-Pierre Allain, Pablo Rubinstein, Michael Mirabile, Sharon Czelusniak, Michael Leuther, Louis Baker, and Alvin E. Friedman-Kien* 575
- Identification of Normal Human Peripheral Blood Monocytes and Liver as Sites of Synthesis of Coagulation Factor XIII a-Chain *Laurie J. Weisberg, Donny T. Shiu, Paul R. Conkling, and Marc A. Shuman* 579
- An ~300 bp Deletion Involving Part of the 5' β -Globin Gene Region Is Observed in Members of a Turkish Family With β -Thalassemia *J.C. Diaz-Chico, K.G. Yang, A. Kutlar, A.L. Reese, M. Aksoy, and T.H.J. Huisman* 583
- Bone Marrow Transplantation for Patients With Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia
S.J. Forman, M.R. O'Donnell, A.P. Nademanee, D.S. Snyder, P.J. Bierman, G.M. Schmidt, J.L. Fahey, A.S. Stein, P.M. Parker, and K.G. Blume 587
- Use of Laser-UV for Inactivation of Virus in Blood Products *Kristina N. Prodouz, Joseph C. Fratantoni, Elizabeth J. Boone, and Robert F. Bonner* 589
- The 3' Ends of the Deletions of Spanish $\delta\beta^0$ -Thalassemia and Black HPFH 1 and 2 Lie Within 17 Kilobases
C. Camaschella, A. Serra, G. Saglio, M. Baiget, N. Malgaretti, R. Mantovani, and S. Ottolenghi 593
- Deposition of Autofluorescent Eosinophil Granules in Pathologic Bone Marrow Biopsies *Michael K. Samoszuk and Froilan P. Espinoza* 597

SPECIAL DEPARTMENTS

- Correspondence 600
- Announcements 602
- Information for Contributors xiii, xiv

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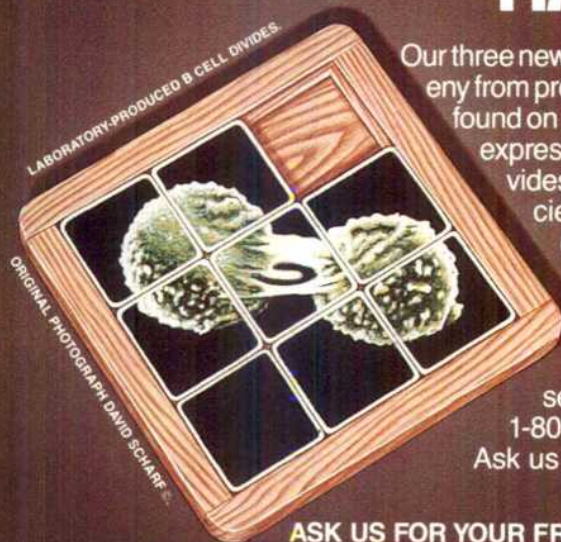
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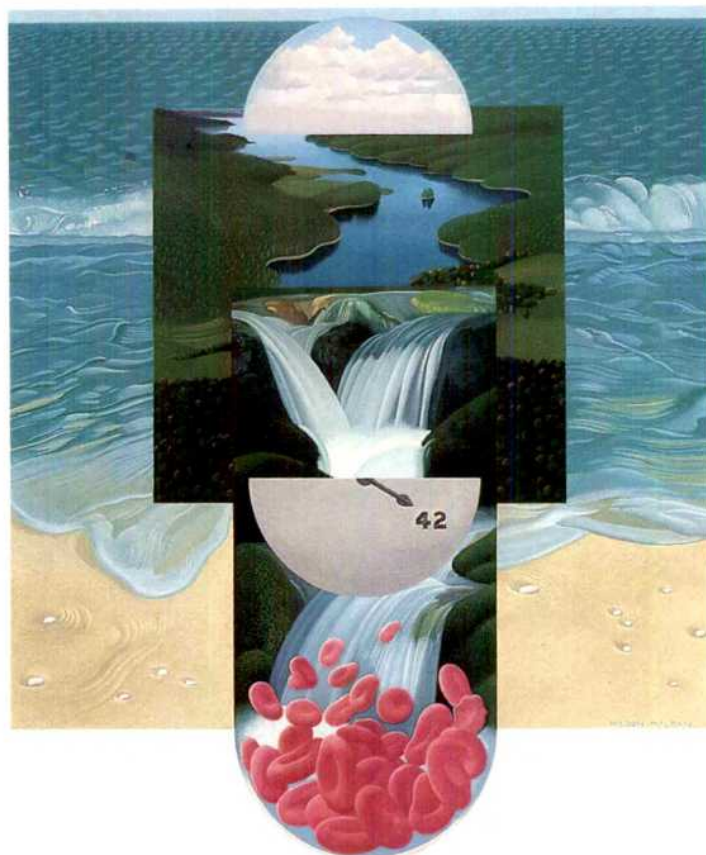
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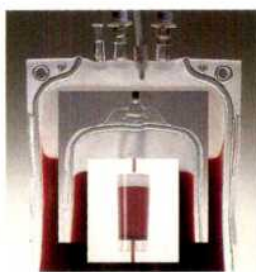


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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. *Br J Haematol* 38:161, 1978

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7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. *Clin Res* 23:271A, 1975 (abstr)

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8. Seeler RA: Sickle cell anemia monthly variations. *Blood* 47:879, 1976 (letter)

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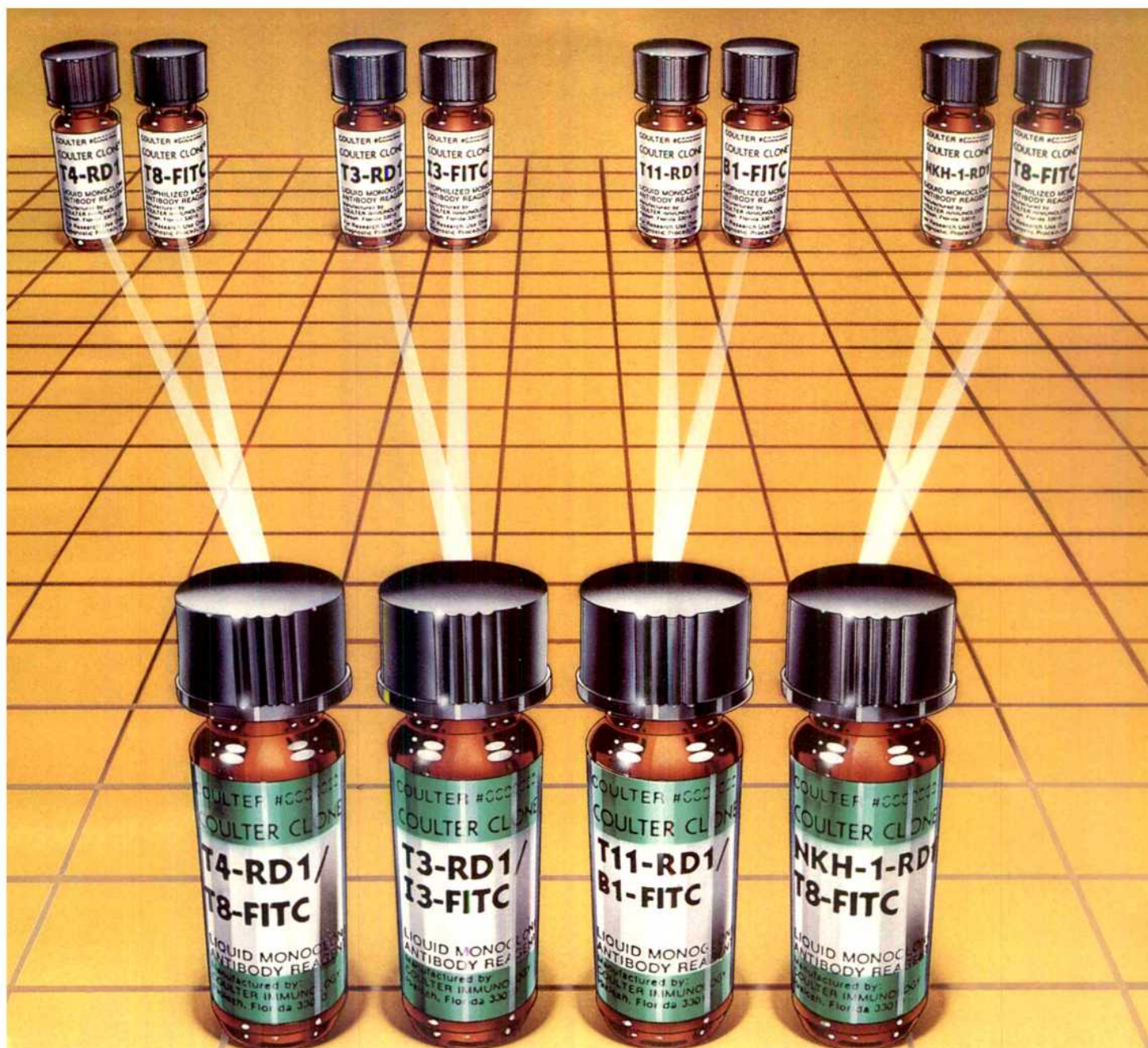
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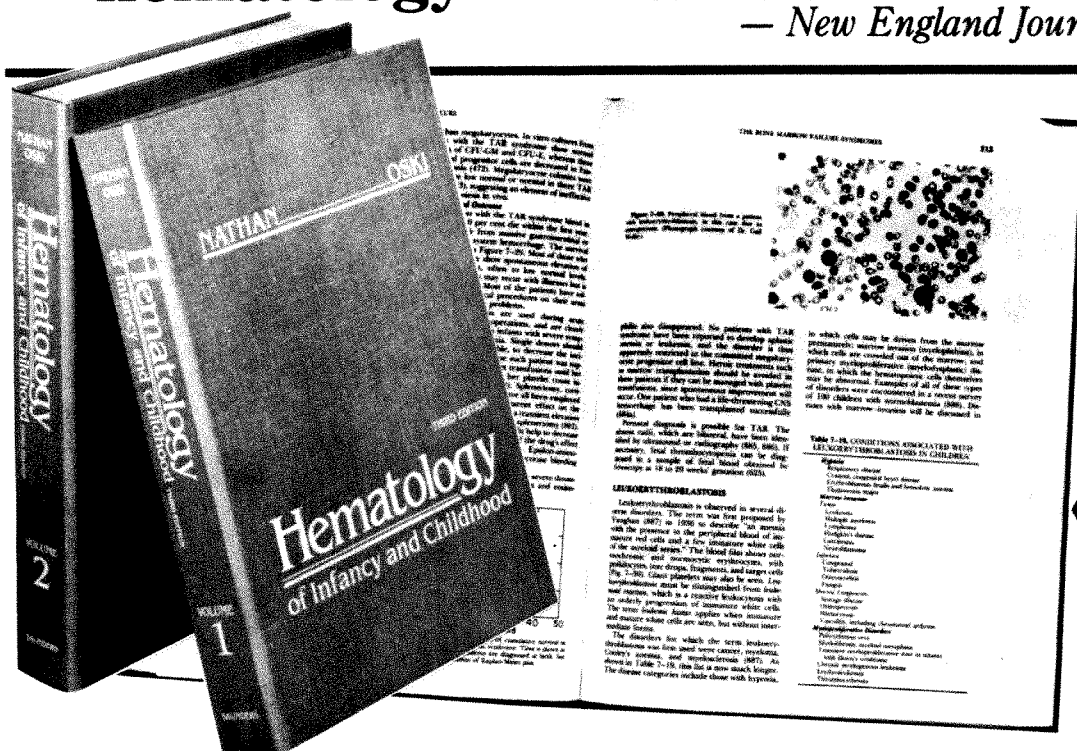
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INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M² every 8 hours (750 mg/M²/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.^{1,2,3}

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.⁴

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.⁵

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.⁶

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M²) over an hour was associated with a lower frequency—4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used

in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax (acyclovir sodium) sterile powder is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M² at a constant rate over 1 hour, every 8 hours (750 mg/M²/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M ²)	Dose (mg/kg)	Dosing Interval (hours)
> 50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, *Lancet* (1983): 1389-1392, Jun. 27, 1981. 2. Wade, *et al.*, *Ann. Intern. Med.* 96(3): 265-269, Mar. 1982. 3. J.D. Meyers, *et al.*, *Am. J. Med.* 73(1A): 229-235, Jul. 20, 1982. 4. Data on file, Burroughs Wellcome Co. 5. A. Mindel, *et al.*, *Lancet* (1982): 697-700, Mar. 27, 1982. 6. Z.M. Naib, *et al.*, *Cancer Res.* 33:1452-1463, 1973.



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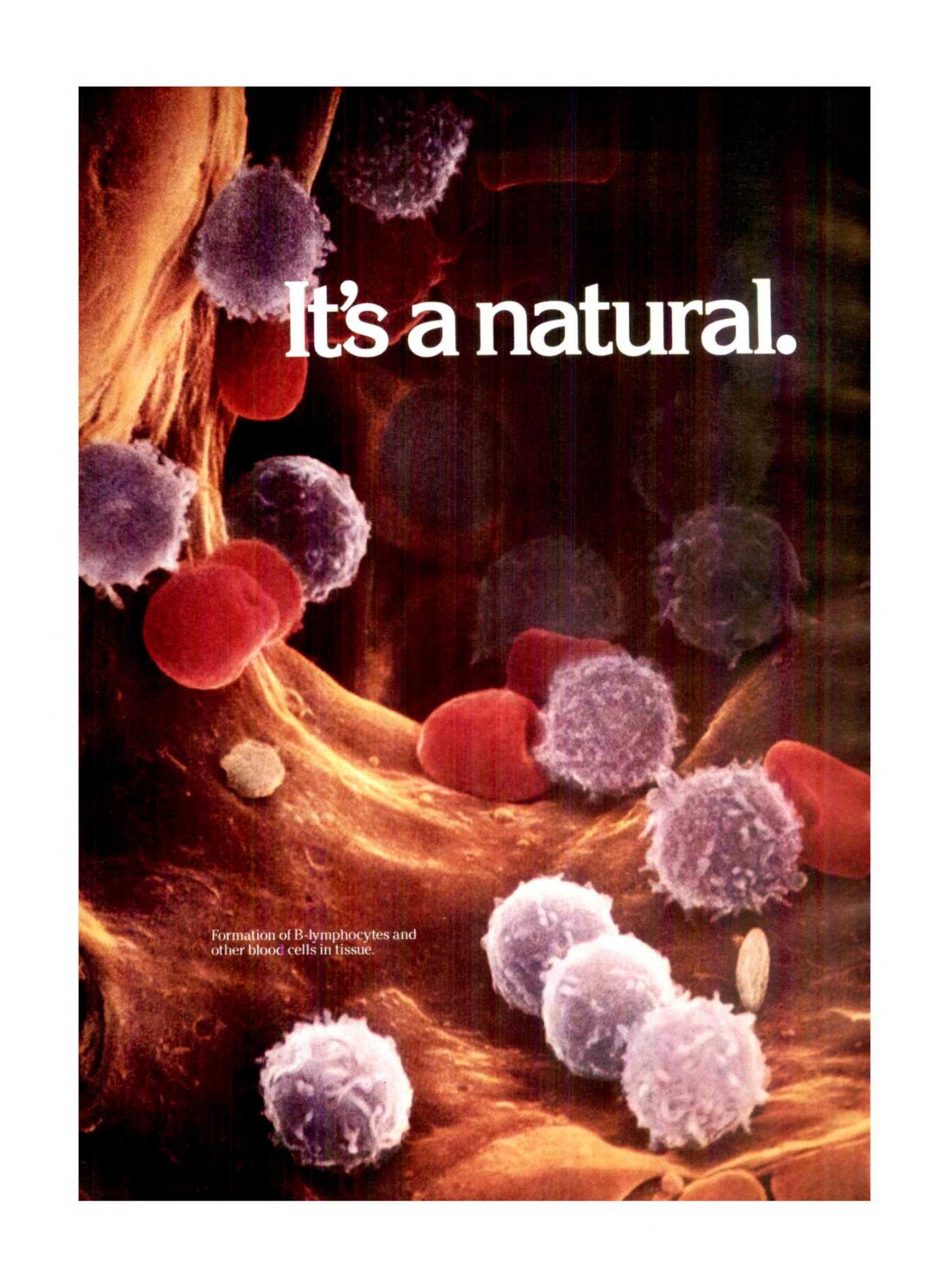
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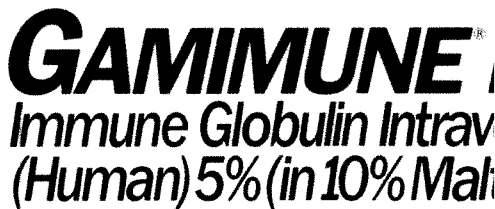
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1. Gamimune® N Product License Application.

2. Lundblad JL, Mitra G, Sternberg MM, et al. Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986; 8(suppl 4): S382-S390.

3. Mitra G, Wong MF, Mozen M, et al. Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986; 26:394-397.

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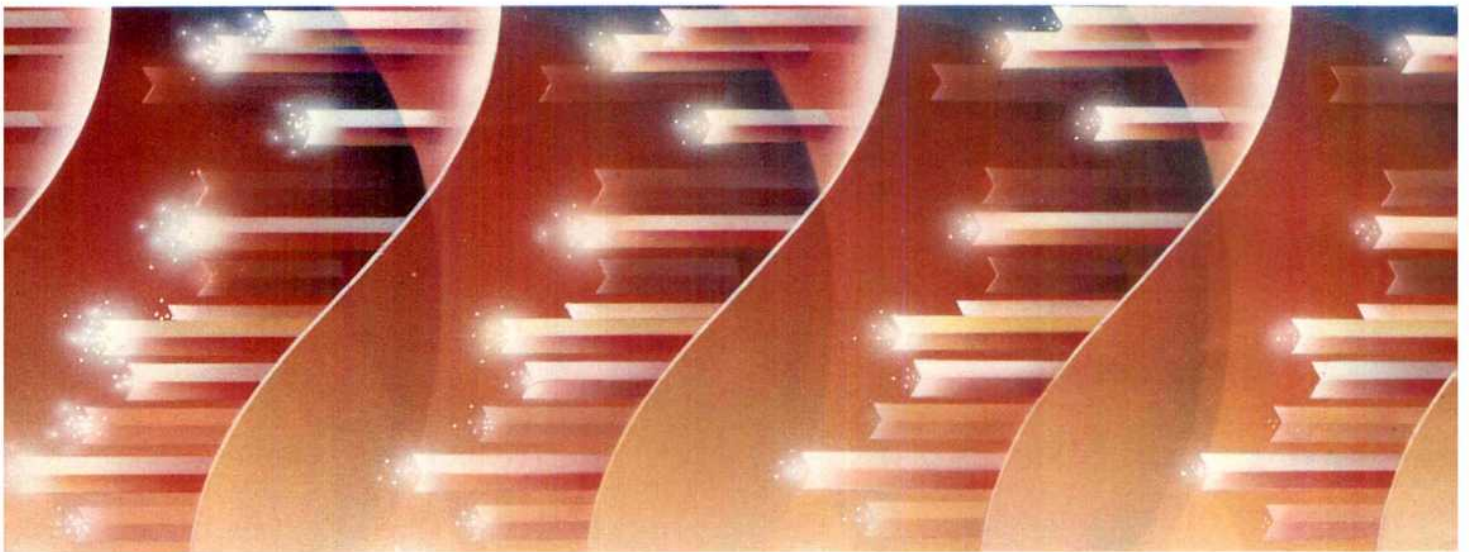
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REVIEW ARTICLE

The Pathophysiology of the Prethrombotic State in Humans: Insights Gained From Studies Using Markers of Hemostatic System Activation

By Kenneth A. Bauer and Robert D. Rosenberg

Numerous investigators have postulated that a hypercoagulable state exists in humans for a period of time before the development of thrombotic episodes. A clear biochemical definition of the prethrombotic state, however, has proved elusive due in part to the lack of reliable techniques for monitoring pertinent changes in blood coagulability. Based on recent advances in our knowledge of the biochemistry of the coagulation system, a series of highly sensitive and specific immunochemical tools has been developed that can quantitate the activities of various steps of the hemostatic mechanism in vivo at the subnanomolar level. We have established assays for F_{1+2} and the protein C activation peptide, which measure the cleavage of the prothrombin molecule by factor Xa and the scission of protein C by the thrombin-thrombomodulin complex, respectively. Nossel and coworkers had previously constructed similar assays for fibrinopeptide A (FPA) and fragment B β 1-42, which monitor the cleavage of fibrinogen by thrombin and the proteolysis of fibrin I by plasmin, respectively. Substantial elevations in the levels of these markers have been found in patients with disseminated intravascular coagulation and many subjects with acute deep venous thrombosis. The F_{1+2} and FPA assays have

been used to demonstrate that significant increments in factor Xa activity but not thrombin activity regularly occur in the blood of nonanticoagulated individuals with congenital deficiencies of antithrombin or protein C. These two disorders are known to be correlated with the subsequent development of thrombosis. Patients with protein C deficiency have also been noted to have significantly reduced plasma levels of protein C activation peptide. By using the immunoassays for FPA and B β 1-42 in studies of postoperative patients, it has been shown that an imbalance between the procoagulant action of thrombin and the anticoagulant effect of plasmin on fibrin I polymer may induce an acquired thrombotic diathesis. Finally, we have recently demonstrated that prothrombin activation as measured by the F_{1+2} assay is suppressed by oral anticoagulants in the blood of patients with thrombotic diatheses. These investigations suggest that these assay techniques can be used to improve our understanding of the hypercoagulable state as well as to develop more effective treatment strategies for the prevention of thromboembolic events.

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THE COAGULATION CASCADE is composed of a series of linked proteolytic reactions. At each stage of the mechanism, a parent zymogen such as prothrombin or factor X is converted to a corresponding serine protease that is responsible for a subsequent zymogen-serine protease transition. In most instances, protein cofactors such as factor V or factor VIII are present and can be activated by serine proteases and then possess the ability to bind the aforementioned reactants to specific cell surfaces. These natural surfaces include platelets, WBC, or endothelial cells. This process usually leads to a dramatic acceleration as well as a partial localization of the reactions. The result of these transformations is the generation of thrombin, which is able to act upon fibrinogen and platelets to produce the hemostatic plug.

For coagulation system enzymes to be generated at any significant rate, a zymogen, a cofactor, and a converting enzyme must form a multimolecular complex on a natural surface. These transformations are suppressed if the converting enzyme is inhibited, the protein cofactor destroyed, or the surface receptors that are essential for the assembly of the macromolecular complex sequestered. Previous methods for

monitoring these processes in the clinic have been directed at measuring the levels of zymogens (ie, factor X, prothrombin, protein C), inhibitors (ie, antithrombin), or substrates (ie, factor VIII, factor V, fibrinogen) of the hemostatic system. These molecular species are present in large excess within the blood, and only a small percentage of the zymogens are converted to active enzymes under in vivo conditions. Thus, attempts to monitor thrombotic or prethrombotic states by measuring the ambient levels of zymogens or inhibitors or by

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determining their catabolic rates within the circulatory system are unlikely to be successful in most instances. Similarly, clotting assays (ie, partial thromboplastin time) that are carried out under arbitrary *in vitro* conditions have not proved to be particularly useful in the assessment of hemostatic system hyperactivity. In addition, neither of these methodologies reflects the endogenous anticoagulant properties of the vascular endothelium.

The enzymes generated by the coagulation cascade are not directly available for quantitation because they are evanescent species that are rapidly neutralized by naturally occurring protease inhibitors. Faced with these obstacles, we have focused our attention on the development of a series of highly sensitive and specific radioimmunoassays for peptides that are liberated with the activation of hemostatic system zymogens *in vivo*. These by-products of zymogen transformation are stable entities with finite half-lives in the circulation. Assays for the F_{1+2} fragment and the protein C activation peptide have been established that measure the cleavage of the prothrombin molecule by factor $Xa^{1,2}$ (Fig 1) and the scission of protein C by the thrombin-thrombomodulin complex,³ (Fig 2) respectively. Nossel and coworkers⁴⁻⁶ had previously constructed immunoassays for fibrinopeptide A and the $B\beta$ 1-42 fragment that monitor the proteolysis of fibrinogen by thrombin (Fig 1) and fibrin I by plasmin, respectively. All individuals exhibit measurable amounts of these markers under normal conditions, and substantial elevations have been noted in patients with disseminated intravascular coagulation, deep venous thrombosis, pulmonary emboli, etc.²⁻⁷ In the following sections we present the data from several clinical studies in which the aforementioned assay techniques have been used to biochemically define the prethrombotic state in patient populations known to suffer from disorders characterized by defective regulation of thrombin generation. We will also present results supporting the view that an imbalance between the procoagulant action of thrombin and the anticoagulant effect of

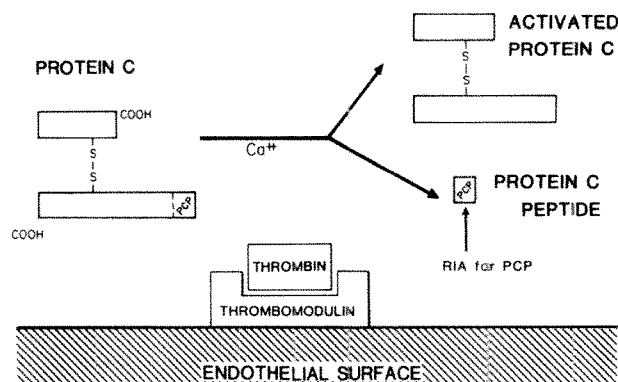


Fig 2. Radioimmunoassay (RIA) for the protein C activation peptide (PCP). Human protein C consists of a heavy chain and light chain that are joined by a single disulfide bridge. During the activation of human protein C by thrombin bound to thrombomodulin on the surface of vascular endothelial cells, an $Arg_{12}-Leu_{13}$ bond is cleaved at the amino-terminal end of the heavy chain of the protein, which releases a small dodecapeptide of approximate molecular weight 1,400. The quantitation of protein C activation in humans has been accomplished by devising a radioimmunoassay for this protein C activation peptide.

plasmin on fibrin I polymer may lead to a thrombotic diathesis in situations where substantial thrombin-mediated proteolysis of fibrinogen has already occurred. The knowledge obtained can then be used to provide a conceptual basis for understanding how these immunochemical tools might be used in more complex situations where it may not be possible to readily define the major molecular defect responsible for thrombotic phenomena in a given individual. No attempt has been made to consider methods for the *in vivo* assessment of platelet activation in this review.

A MODEL FOR THE REGULATION OF THROMBIN GENERATION

Based upon recent advances in our knowledge of the hemostatic mechanism, we will first construct a tentative

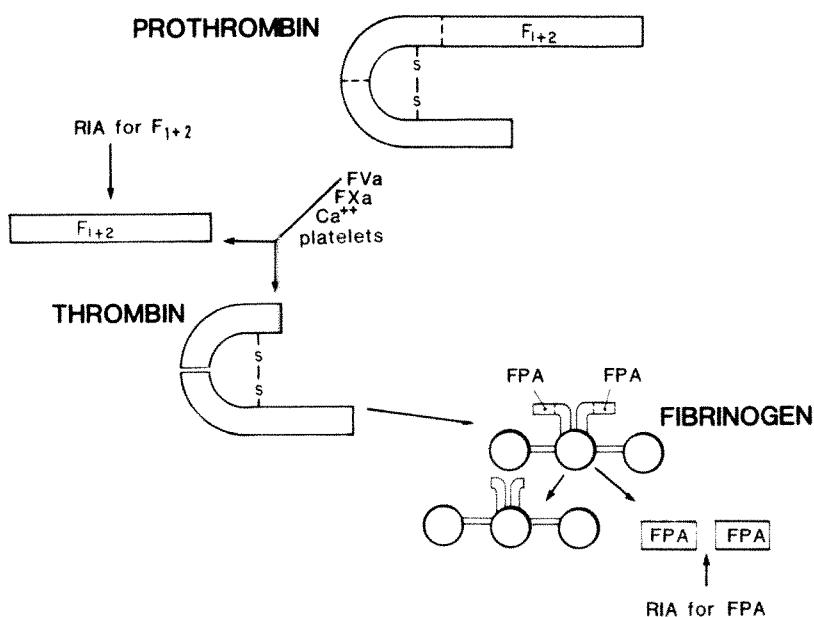


Fig 1. Radioimmunoassays (RIAs) for F_{1+2} and fibrinopeptide A (FPA). The conversion of prothrombin to thrombin takes place under physiological conditions in the presence of factor Xa, factor Va, calcium ions, and platelets. During this process, two peptide bonds in the zymogen are scissored. Cleavage at $Arg_{273}-Thr_{274}$ results in the release of the inactive F_{1+2} fragment from the amino terminus of prothrombin, and a second bond scission at $Arg_{322}-Ile_{323}$ results in the generation of the two-chain serine protease thrombin. Once this enzyme is evolved, the initial step in the conversion of fibrinogen to fibrin results in the liberation of FPA.

model for the regulation of thrombin generation under *in vivo* conditions (Fig 3) with which to interpret the results of our clinical investigations. We hypothesize that small amounts of factor Xa as well as factor Va are continuously formed within the vascular system. These components are able to bind to specific platelet receptors to form the prothrombinase complex and produce small amounts of thrombin. Natural anticoagulant mechanisms act to oppose the generation of the latter enzyme.^{8,9} The endogenous heparan sulfate-antithrombin and protein C-thrombomodulin mechanisms function to inhibit serine proteases (ie, factor Xa, thrombin) and inactivate activated cofactors of the coagulation cascade (ie, factor VIIIa, factor Va), respectively. Hence, these processes oppose the formation of the prothrombinase complex as well as the action of thrombin. The ability of these two regulatory systems to limit the production of thrombin on the surface of platelets and endothelial cells should determine whether activation of platelets takes place within the circulatory system. If platelet activation occurs, prothrombin is brought into close proximity with the factor Va-factor Xa complex on the platelet surface, and significant amounts of thrombin can be generated that might gradually overwhelm the natural anticoagulant mechanisms.

CLINICAL STUDIES OF THE INHERITED THROMBOTIC DISORDERS

The aforementioned model for the regulation of thrombin generation predicts that the prethrombotic state can be defined as an imbalance between the procoagulant and

natural anticoagulant mechanisms that eventually leads to the development of overt thrombotic phenomena. To examine this hypothesis, we have studied the biochemical alterations in hemostatic system activity that arise in patients with congenital reductions in the levels of antithrombin and protein C. These relatively homogenous inherited disorders should cause a prolonged decrease in the activity of the endogenous heparan sulfate-antithrombin or protein C-thrombomodulin mechanisms before the occurrence of thrombotic disease. Indeed, these patients often do not become symptomatic until their early twenties, and increasing numbers of individuals experience venous thromboembolic events with advancing age.^{10,11}

Radioimmunoassays for the prothrombin activation fragment F_{1+2} and fibrinopeptide A were used to quantitate the levels of free factor Xa and thrombin, respectively, within the blood of 22 asymptomatic individuals not receiving anticoagulants from eight separate kindreds with congenital antithrombin deficiency.¹² The median age of this cohort was 22. Only seven of these patients had a prior history of an overt thrombotic episode, and at least two other members of each family had exhibited deep vein thrombi or pulmonary emboli. Our studies demonstrated that the plasma F_{1+2} levels were significantly elevated in almost all patients with this disorder ($P < .001$) (Table 1) whereas the concentrations of fibrinopeptide A were not substantially altered in these individuals as compared with normal controls. Clearance studies with ^{131}I - F_{1+2} revealed that the metabolic behavior of this component was identical in antithrombin-deficient subjects and normal individuals. These findings suggest that patients with this inherited thrombotic disorder exhibit

Fig 3. A model for the regulation of thrombin generation *in vivo* by the natural anticoagulant mechanisms of the endothelium. Factor Xa, factor Va, protein S, prothrombin activation fragment F_{1+2} , fibrinopeptide A, antithrombin, and activated protein C inhibitor are designated as FXa, FVa, S, F_{1+2} , FPA, AT, and activated protein C inhibitor, respectively. Thrombin may be inactivated by forming 1:1 stoichiometric complexes with its major physiological inhibitor, antithrombin, thereby resulting in the formation of thrombin-antithrombin complexes (thrombin-AT). Activated protein C can be neutralized by the activated protein C inhibitor, which results in the generation of activated protein C-inhibitor complexes.

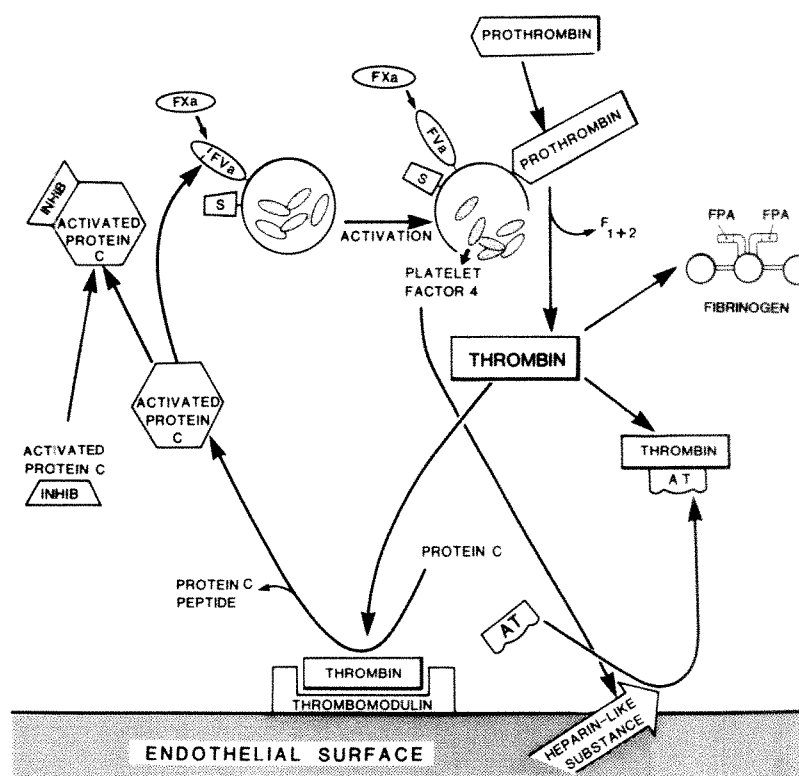


Table 1. Levels of F_{1+2} in Patients With Hereditary Deficiencies of Antithrombin or Protein C

Patient Population (No. of Subjects Evaluated)	F_{1+2} (nmol/L) (Mean $\pm$ SD)
Not receiving anticoagulants	
Antithrombin-deficient subjects (22)	$3.91 \pm 1.13^\dagger$
Protein C-deficient subjects (23)	$2.54 \pm 1.11^\ddagger$
Age-matched normal subjects without an inherited or acquired thrombotic diathesis (31)	1.51 ± 0.68
Receiving oral anticoagulants	
Antithrombin-deficient subjects (12) (PT ratio* = 1.85 ± 0.41)	$0.714 \pm 0.26\$$
Protein C-deficient subjects (22) (PT ratio* = 1.78 ± 0.32)	0.205 ± 0.25
Subjects without an inherited thrombotic diathesis (23) (PT ratio* = 1.74 ± 0.32)	$0.231 \pm 0.24\parallel$

*PT (prothrombin time) ratio = PT of patient/PT of normal plasma pool and is expressed as the mean $\pm$ SD.

$^\dagger P < .0001$. Antithrombin deficient subjects not receiving oral anticoagulants v age-matched normal subjects.

$^\ddagger P < .0005$. Protein C deficient subjects not receiving oral anticoagulants v age-matched normal subjects.

$\$ P < .01$. Antithrombin deficient subjects receiving oral anticoagulants v anticoagulated subjects without an inherited thrombotic diathesis or protein C deficient subjects on oral anticoagulants.

$\parallel P < .0001$. Subjects without an inherited thrombotic diathesis receiving oral anticoagulants v age-matched normal subjects.

increased concentrations of factor Xa that lead to excessive activation of prothrombin but that the resulting thrombin is neutralized before it can cleave fibrinogen to any significant extent. We also infused purified antithrombin concentrate into several affected individuals and monitored the response of their hemostatic mechanisms. The data obtained showed that elevations in the levels of protease inhibitor led to a decrease in the abnormally high concentrations of F_{1+2} . It was also observed that the levels of this marker remained at a relatively constant normal value as antithrombin levels steadily declined from their peak concentrations postinfusion but started to rise toward the high preinfusion value when the plasma concentrations of the inhibitor dropped below ~70% of normal.¹²

Marcum et al¹³⁻¹⁵ have provided biochemical, cell biologic, as well as physiological evidence that heparinlike proteoglycans intimately associated with the vascular endothelium can accelerate hemostatic enzyme-antithrombin complex formation via a process that is identical to that of commercial heparin. In addition, Carlson and coworkers¹⁶ have noted that metabolic turnover studies in humans with radiolabeled antithrombin are consistent with a model in which ~10% of the total pool of antithrombin is associated with a noncirculating vascular compartment that probably represents the heparinlike proteoglycans of the vessel wall. It therefore appears likely that a small fraction of plasma antithrombin is normally bound to the unique heparinlike proteoglycans of the vessel wall in an activated conformation. This pool of protein probably represents the physiologically relevant protease inhibitor population, whereas the same component is of minimal functional relevance when free within the blood.

Therefore, we surmise that antithrombin-deficient patients have an insufficient plasma level of protease inhibitor to saturate the heparinlike proteoglycans of the vessel wall and hence cannot generate sufficient activated protein to suppress factor Xa formed within the circulatory system. This supposition would provide an explanation for the decrease in F_{1+2} values that occurs when the concentrations of antithrombin are ~70% of normal and for the subsequent plateau of this parameter despite ambient levels of protease inhibitor that ranged between 70% and 250% of normal.¹²

We have also used the specific radioimmunoassays for F_{1+2} , fibrinopeptide A, and protein C activation peptide to quantitate the production and action of thrombin as well as the activation of protein C within the blood of 23 asymptomatic individuals not receiving anticoagulants from 13 kindreds with an isolated congenital deficiency of protein C.¹⁷ The median age of this cohort was 37, and nine of these patients had previously sustained at least one thrombotic event. The mean concentrations of both protein C and protein C activation peptide were reduced to about 50% of normal in the members of this population. The mean level of F_{1+2} was significantly elevated by almost twofold in subjects with protein C deficiency ($P < .0005$) (Table 1) whereas the mean concentration of fibrinopeptide A was only minimally raised as compared with an age-matched control group. The metabolic behavior of ^{131}I - F_{1+2} was found to be similar in protein C-deficient patients with elevated F_{1+2} measurements and normal individuals. Several of the 23 subjects, however, had normal plasma F_{1+2} concentrations, and our results suggest that the extent of hemostatic system activation as measured by this assay in heterozygotes with protein C deficiency may be much more variable than that in patients with congenital antithrombin deficiency. Data similar to that outlined for the antithrombin-deficient patients have been obtained in a more limited number of kindreds with protein S deficiency. Based upon these data, it would appear that malfunction of the endogenous heparan sulfate-antithrombin or protein C-thrombomodulin mechanisms will result in excessive generation of thrombin well before the development of overt thrombotic disease.

In this regard, it is critical to emphasize that many asymptomatic patients with the aforementioned deficiency states exhibit hemostatic system hyperactivity for prolonged periods of time but the documented occurrence of thrombotic complications within this population is episodic. The biochemical processes that are responsible for the conversion of this hypercoagulable state into an actively thrombotic one are poorly understood at the present time. A variety of events could acutely depress the effective functioning of the endogenous heparan sulfate-antithrombin or protein C-thrombomodulin mechanisms. These would include minor alterations in the plasma concentrations of antithrombin or protein C and transient reductions in the levels of thrombomodulin or heparinlike proteoglycans on the surface of endothelial cells. In addition, a decrease in the free plasma protein S concentration via changes in the interaction of this component with the C4b-binding protein may reduce the ability of activated protein C to inactivate factors VIIIa and Va.^{18,19}

POSTULATED MOLECULAR DEFECTS IN COMPLEX THROMBOTIC DISORDERS

Despite our uncertainties about various aspects of the pathophysiology of congenital deficiencies of antithrombin, protein C, and protein S, it is clear that many patients with these disorders biochemically exhibit a true prethrombotic state, ie, they have excessive production of factor Xa enzymatic activity with insufficient generation of thrombin to convert significant amounts of fibrinogen to fibrin. It remains to be determined whether elevations in the concentration of F_{1+2} will have predictive value in determining the subsequent development of a thrombotic event in these populations. The vast majority of thrombotic disorders, however, are not associated with inherited risk factors and are oftentimes associated with acquired conditions, ie, lupus anticoagulant, neoplasia, advancing age, postoperative state, estrogen therapy, obesity, etc. Due to their complex pathophysiology, it seems unlikely that one will be able to implicate isolated abnormalities in the levels of antithrombin, protein C, or protein S as primary etiologic factors for the hypercoagulable syndromes associated with these conditions. The application of immunochemical assays such as that for prothrombin fragment F_{1+2} to such populations might potentially identify those individuals at highest risk for the subsequent development of thrombotic phenomena.

In view of the known association of vascular disease with increasing age, we have conducted a detailed analysis of hemostatic system activity with respect to perturbations induced by aging phenomena.²⁰ None of these individuals exhibited an immunochemical deficiency of either antithrombin or protein C. The levels of F_{1+2} were quantitated in the plasma of 199 healthy males between the ages of 42 and 80 and demonstrated a highly significant positive correlation with increasing age ($P < .0001$). The elevations in the concentration of this component in aged patients were not due to diminished clearance of the fragment. Significant elevations in the levels of fibrinopeptide A ($P < .01$) and protein C activation peptide ($P < .002$) were also observed with advancing age. These results indicate that many apparently normal males of increasing age exhibit a biochemical defect that denotes the presence of an acquired prethrombotic state.

One can use the aforementioned model (Fig 1) to suggest a variety of potential molecular defects in the natural anticoagulant mechanisms that might lead to elevated F_{1+2} levels in populations with acquired risk factors for the eventual development of thrombotic disease. For example, decreased synthesis of heparinlike molecules by endothelial cells or increased release of platelet factor 4 by platelets are likely to significantly reduce the activity of the endogenous heparan sulfate-antithrombin mechanism without altering the levels of the protease inhibitor. In addition, decreased production of thrombomodulin by endothelial cells, decreased activity of the postulated receptor for activated protein C on platelets, decreased levels of functional protein S, or increased levels of the protein C inhibitor²¹ might also dramatically reduce the activity of the protein C-thrombomodulin mechanism without altering the concentrations of protein C. Furthermore,

decreased generation of prostaglandin I_2 by endothelial cells, decreased numbers of receptors for this prostaglandin on platelets, or decreased ability of platelets to respond to the suppressive action of this prostaglandin would allow the bound prothrombinase complex to activate increasing amounts of prothrombin.²²

Other patients who experience multiple thrombotic episodes may exhibit an increased production of thrombin within the circulatory system whose actions cannot be suppressed by the normal functioning of the natural anticoagulant mechanisms. The potential molecular defects that could lead to this situation include excessive exposure of tissue factor by endothelial cells²³ or WBC, increased numbers of receptors for factor IX/IXa or factor X/Xa on endothelial cells,^{24,25} augmented numbers of sites for factor Va on platelets^{26,27} or endothelial cells, or increased capacity of platelets or endothelial cells to bring prothrombin into close approximation with the prothrombinase complex, etc. At the present time, we have little real knowledge about this set of potential abnormalities.

A MODEL FOR THE REGULATION OF THROMBIN ACTION ON FIBRIN I POLYMER

The conversion of fibrinogen to fibrin by thrombin initially results in the formation of fibrin I monomer and the release of fibrinopeptide A (Fig 4). Fibrin I monomer is able to polymerize, and thrombin can then cleave this polymer, thereby resulting in the generation of fibrin II and the release of fibrinopeptide B. Tissue-type plasminogen activator is able to bind to fibrin, which allows plasminogen to be activated to plasmin at an increased rate. The action of the serine protease plasmin on fibrin I polymer results in the production of fragment X along with fragments from the α and β chains ($B\beta$ 1-42). Plasmin can subsequently convert fragment X to soluble degradation products.

MOLECULAR DEFECTS IN THE FIBRINOLYTIC MECHANISM

Dysfunction of the fibrinolytic system may also play an important role in thrombus formation. Although investigators have identified a few individuals with inherited abnormalities of the fibrinolytic mechanism and recurrent venous thromboembolism,^{28,29} we consider the clinical association between these two conditions to be less convincing than what has been observed in many kindreds with deficiencies of antithrombin, protein C, or protein S. This relative lack of success in the identification of fibrinolytic mechanism defects may be related to inadequacies in the laboratory methodologies that have been available for the detection of clinically relevant abnormalities.

It has been tacitly assumed that thrombotic phenomena observed in patients with fibrinolytic system defects are due to their reduced ability to lyse small fibrin clots and prevent proximal extension. Nossel³⁰ proposed an alternative view that an imbalance between the relative rates at which thrombin and plasmin split the $B\beta$ chain of fibrin I polymer could determine the occurrence of venous thrombosis. Owen et al³¹ have used radioimmunoassays for fibrinopeptide A

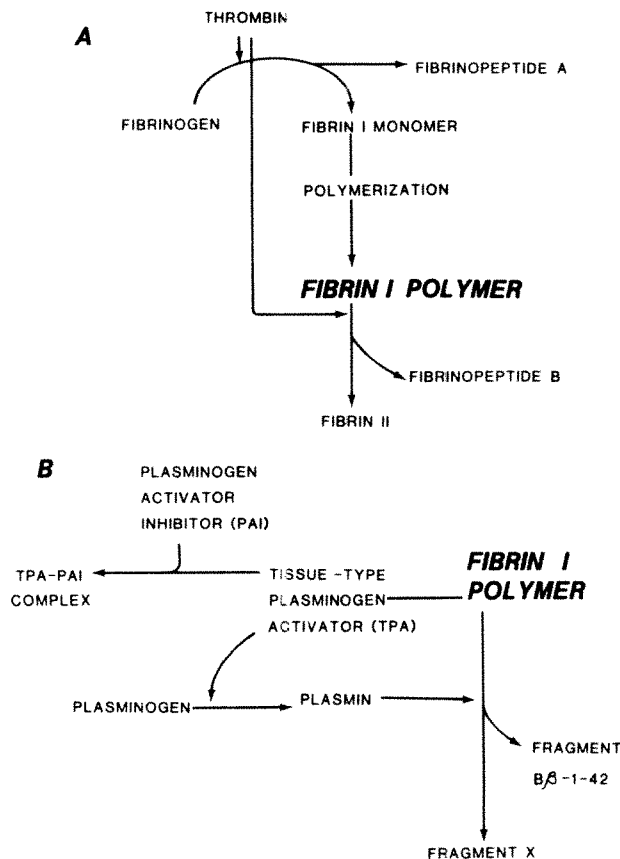


Fig 4. A model for the regulation of thrombin action on fibrin I polymer in vivo. (A) The conversion of fibrinogen to fibrin II by thrombin. (B) The fibrinolytic activity of human plasma is determined primarily by the presence of tissue-type plasminogen activator (TPA) and its inhibitor (PAI), which are released from vascular endothelial cells. TPA is able to bind to fibrin I polymer, which allows plasminogen to be activated to plasmin at an increased rate.

and B β 1-42 (measured as thrombin-increasable fibrinopeptide B) to quantitate thrombin and plasmin activity, respectively, in patients under craniotomy. Venous thrombosis was documented by ^{125} I-fibrinogen leg scanning. The results obtained indicated that individuals in whom thrombi developed, when compared with those who did not suffer from this complication, exhibited levels of fibrinopeptide A that were considerably greater than the concentrations of B β 1-42 during the four days preceding the onset of this disorder. These observations suggest that thrombosis occurs at a time when thrombin action is enhanced relative to that of plasmin.

The precise molecular defects that are responsible for these phenomena are not currently well understood. Fibrinolysis is initiated by plasminogen activators that convert plasminogen to the active enzyme plasmin by limited proteolysis. Plasmin is able to degrade fibrin to soluble products or alternately may be inactivated by forming 1:1 stoichiometric complexes with its major physiological inhibitor α_2 -antiplasmin.³² The fibrinolytic activity of human plasma is determined primarily by the presence of tissue-type plasminogen activator and its inhibitor, which are released from vascular

endothelial cells.^{33,34} Immunochemical methods for the quantitation of tissue-type plasminogen activator and functional assays for the inhibitor have recently been applied to the study of patients with documented venous thromboembolism. These studies have suggested that defective synthesis or release of tissue-type plasminogen activator as well as an increased concentration of the inhibitor of this serine protease may be important pathogenetic factors in some of these individuals.^{35,36} It should also be noted that reduced fibrinolytic activity due to increased plasma levels of a rapid inhibitor of tissue-type plasminogen activator has been found in young survivors of myocardial infarction.³⁷

SUPPRESSION OF PROTHROMBIN ACTIVATION BY ORAL ANTICOAGULANTS

The radioimmunoassay for F₁₊₂ has recently been used to study patients without an inherited thrombotic diathesis who were stably anticoagulated with sodium warfarin.³⁸ Our results demonstrate that prothrombin activation as measured by this assay is markedly suppressed in individuals with prothrombin time ratios (prothrombin time of patient/prothrombin time of normal plasma pool) greater than 1.5 (Table 1). The reduction in the plasma concentration of this component was not due to decreased immunoreactivity or rapid clearance of partially γ -carboxylated forms of the fragment. Thus, measurement of F₁₊₂ levels should serve as an accurate marker of factor Xa activity upon prothrombin in this patient population. The substantial suppressive effect of standard warfarin schedules suggests that a less intense

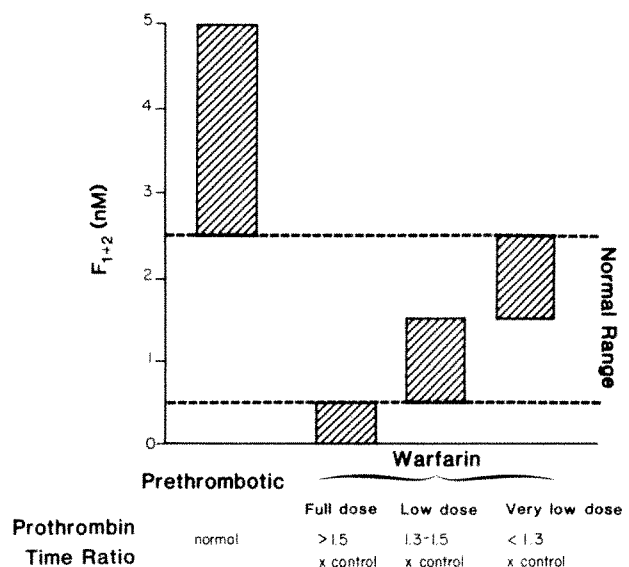


Fig 5. A schematic diagram demonstrating the response of the hemostatic mechanism as measured by the F₁₊₂ radioimmunoassay to oral anticoagulation schedules of varying intensity as measured by the prothrombin time ratio (prothrombin time of patient/prothrombin time of normal plasma pool). Patients enrolled in this research protocol had thrombotic diatheses and elevated plasma F₁₊₂ levels before the initiation of warfarin therapy. All subjects had been free of thrombotic complications for at least 3 months before study. This figure is shown for illustrative purposes only and is not intended to depict the exact results obtained in all patients studied.

anticoagulation regimen might lead to a significant reduction of elevated F_{1+2} levels into the normal range rather than to subnormal concentrations. We therefore carried out studies of the response of the hemostatic mechanism to a very low intensity anticoagulation program (prothrombin time ratio less than 1.3) in five patients with a prior history of thrombotic disease. All of these individuals had elevated plasma F_{1+2} levels before the institution of warfarin therapy. The administration of low doses of the drug led to a decrease in the concentration of this marker into the normal range at a mean prothrombin time ratio that was essentially indistinguishable from untreated subjects. A schematic diagram of the results of this investigation is shown in Fig 5.

We also evaluated the effects of warfarin on factor Xa enzymatic activity in individuals with hereditary deficiencies of antithrombin or protein C who were chronically receiving warfarin. At equivalent levels of intensity of oral anticoagulation, the mean plasma F_{1+2} level in patients with antithrombin deficiency was significantly elevated as compared with the subjects with protein C deficiency or with the group of anticoagulated persons without an inherited thrombotic disorder ($P < .01$) (Table 1). We therefore conclude that the effect of warfarin on hemostatic system activation is modulated by the endogenous heparan sulfate-antithrombin mechanism. These data also imply that the chronic administration of warfarin leads to a dominant anticoagulant effect on the vitamin K-dependent procoagulant factors of the

hemostatic system as compared with the antagonistic effect of the drug on the protein C anticoagulant mechanism when the endogenous heparan sulfate-antithrombin anticoagulant pathway is functioning normally.

Recent clinical studies have demonstrated that low-intensity regimens of warfarin provide prophylaxis against venous thrombosis,³⁹ but the minimal level of oral anticoagulants required in a given patient remains to be determined. The F_{1+2} assay may therefore be useful in monitoring the in vivo action of low-intensity warfarin therapy in clinical trials designed to establish the lowest amount of medication needed to protect against a thrombotic event.

CONCLUSION

This review has summarized the results of several clinical studies that indicate that a biochemical imbalance between procoagulant and anticoagulant mechanisms can be detected in the blood of humans before the appearance of thrombotic phenomena. The continuing application of biochemical, molecular biologic, and clinical approaches to investigations of the hemostatic system should lead to a greater appreciation of the molecular defects that lead to venous and arterial vascular disorders in humans. It is to be hoped that this new information will allow us to more precisely identify individuals who are entering a clinically relevant hypercoagulable state and intervene with appropriate therapy before the onset of overt thrombotic disease.

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Characterization of the Chemotactic Defect in Polymorphonuclear Leukocytes Exposed to Influenza Virus In Vitro

By Dorothy L. Moore and Elaine L. Mills

The mechanism by which influenza virus interferes with polymorphonuclear leukocyte (PMN) chemotaxis was investigated. Incubation of human PMN with influenza A virus in vitro for 30 minutes significantly decreased PMN migration under agarose in response to N-formyl-methionyl-leucyl-phenylalanine (FMLP) or zymosan-activated serum. Virus-treated PMN tended to aggregate in the under-agarose assay. Aggregation was avoided by using a more dilute PMN suspension in filter assays. Virus treatment significantly decreased migration through 100- μ m thick cellulose nitrate filters but had no effect on migration

through 10- μ m thick polycarbonate filters or on PMN bipolar shape change. Virus was not chemotactic in the polycarbonate filter assay and did not induce shape change in purified PMN. It was concluded that influenza virus did not interfere with the ability of PMN to recognize a chemoattractant, undergo shape change, and move a short distance but did limit the extent of migration. Inhibition could not be explained by chemotactic deactivation, since the virus was not chemotactic.

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INFECTION with influenza virus has been shown to predispose the host to secondary bacterial infection and to interfere with a number of phagocytic cell functions including chemotaxis.¹ Defective monocyte² and polymorphonuclear leukocyte (PMN) chemotaxis^{3,4} have been reported in patients and volunteers infected with influenza A. Defective chemotaxis has also been demonstrated in PMN of chinchillas experimentally infected with influenza A,⁵ as well as in normal human PMN⁶⁻¹⁰ or monocytes^{2,11} incubated in vitro with influenza A or B. The mechanism by which this inhibition occurs is not known. Chemotaxis is a complex process involving the recognition of a chemoattractant, the triggering of a series of biochemical events leading to microtubule assembly, a change to a bipolar shape, orientation of this bipolar cell toward the chemical gradient source, increased adhesiveness and deformability, and movement toward the chemoattractant.¹² In addition, degranulation of specific granules onto the plasma membrane surface occurs in association with a decrease in net surface charge.¹³ In an attempt to understand at what stage influenza virus interferes with this process, we have examined locomotion by migration under agarose and through filters and have assessed the early phases of chemotaxis by evaluating cell shape change. The possibility that the virus itself might be chemotactic was also investigated.

MATERIALS AND METHODS

Preparation of PMN. Human PMN were purified ($\geq 98\%$) from heparinized whole blood of healthy adult volunteers by dextran sedimentation, centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), and hypotonic lysis of residual erythrocytes as described previously.¹⁴ The PMN pellet was washed twice in approximately 30 vol of Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY) containing 0.1% gelatin. PMN were from 12 different donors.

Preparation of virus. Influenza A/Texas/77 (H3N2) virus was harvested from allantoic fluid of infected embryonated hens' eggs,¹⁴ purified by sucrose density gradient ultracentrifugation,¹⁵ suspended in 0.01 mol/L phosphate-buffered saline (PBS), aliquoted and stored at -70°C . Hemagglutination titrations were performed in microtiter plates.¹⁶ Two preparations, with hemagglutination titers of 3,200 and 20,480 HAU respectively, and $\geq 10^{10}$ egg infectivity doses per milliliter were used. Control preparations were made from uninfected allantoic fluid (AF) processed in an identical manner.

Chemotaxis under agarose. Migration under agarose was measured by the method of Nelson et al¹⁷ in Petri plates containing 0.8%

agarose (Type 1, Sigma Chemical Co, St. Louis) and 0.25% gelatin in Eagles Minimal Essential Medium (MEM; Flow Laboratories Inc, McClean, VA). PMN were suspended in MEM containing 0.1% gelatin (MEMg) at a cell concentration of $1.2 \times 10^6/\text{mL}$. Varying amounts of virus or AF were added and the mixtures incubated at 37°C for 30 minutes with constant rotation. PMN were collected by centrifugation, counted in trypan blue to assess viability, resuspended in MEMg at a concentration of $5 \times 10^7/\text{mL}$, and plated in triplicate wells in volumes of 10 μL per well. Ten μL volumes of N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma Chemical Co) at concentrations of 1×10^{-7} or 5×10^{-7} mol/L, 15 μL vol of zymosan-activated serum (ZAS) prepared from pooled human serum¹⁸ or 10 μL MEMg were placed in adjacent wells. The plates were incubated in 5% CO_2 at 37°C for 2½ hours. The cells were then fixed with 2% glutaraldehyde in PBS, the agar was removed, and the plates were stained with Diff-Quick (Harleco, Gibbstown, NJ). Migration distances were measured using an overhead projector and results expressed as the mean migration observed in the triplicate wells.

Chemotaxis through filters. Migration through filters was assayed with a multiwell chemotaxis chamber (Neuro Probe, Inc, Cabin John, MD) using Toyo 100- μ m thick cellulose nitrate filters, pore size 5 μm (Neuro Probe, Inc), and 10- μ m thick polyvinylpyrrolidone-free polycarbonate filters, pore size 5 μm (Nucleopore Corp, Pleasanton, CA).¹⁹

For the 100- μ m thick filters, PMN were treated with virus or AF as for the agarose technique, then suspended in MEMg at a concentration of $1 \times 10^6/\text{mL}$. Twenty-five μL vol of FMLP (1×10^{-8} mol/L to 1×10^{-6} mol/L in MEMg) or MEMg were added to the lower wells of the chamber and 50 μL volumes of PMN to the upper wells. Each test was performed in triplicate in each of two chambers. The chambers were incubated in 5% CO_2 at 37°C , one for one hour and the other for 2½ hours. The filters were removed, fixed in formaldehyde, stained with Meyer's hematoxylin, dehydrated, and cleared in xylene. Migration was quantitated by determining the position of the leading front and the number of cells migrating to a

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Table 1. Effect of Influenza Virus on PMN Migration Under Agarose

Virus per 10 ⁶ PMN	Chemoattractant	Migration (mm)*		% Inhibition*
		Virus-Treated PMN	Control PMN	
100 HAU	FMLP 1 × 10 ⁻⁷ mol/L	1.11 ± 0.37	1.91 ± 0.25†	42 ± 16
	FMLP 5 × 10 ⁻⁷ mol/L	1.84 ± 0.17	2.29 ± 0.20‡	20 ± 9
	ZAS	0.59 ± 0.51	1.41 ± 0.69§	63 ± 13
	None	0.12 ± 0.04	0.20 ± 0.04‡	40 ± 20
10 HAU	FMLP 1 × 10 ⁻⁷ mol/L	1.32 ± 0.19	1.78 ± 0.26†	25 ± 9
	FMLP 5 × 10 ⁻⁷ mol/L	2.00 ± 0.25	2.37 ± 0.36‡	15 ± 7
	None	0.13 ± 0.05	0.14 ± 0.05	—

*Mean ± SD of values from four or five separate experiments with PMN from different donors.

†Difference between virus-treated and control PMN significant at $P < 0.02$.

‡Difference between virus-treated and control PMN significant at $P < 0.05$.

§Difference between virus-treated and control PMN significant at $P < 0.01$.

distance of 60 μ m after incubation for one hour and the number of cells migrating through to the undersurface of the filter after 2½ hours. Three to five high-power fields were examined for each well and the results expressed as the mean values from triplicate wells.

For the 10- μ m filters the procedure was similar except that HBSS was used instead of MEMg in the chambers. Incubation was for one hour. The filter was processed and stained by the method of Harvath et al.¹⁹ The number of cells migrating to the undersurface of the filter was determined. Three high-power fields were counted for each well and the results expressed as the mean counts of triplicate wells.

In experiments to determine whether or not influenza virus was chemotactic, virus or AF was placed in the lower wells and untreated PMN in the upper wells. The 10- μ m polycarbonate filter was used.

Bipolar shape change. Bipolar shape change was investigated using the method of Smith et al.²⁰ Fifty μ L vol of virus or AF were added to 500 μ L vol of PMN (1×10^6 /mL) in HBSS and the mixtures incubated at room temperature or 37°C for 30 minutes. Fifty μ L of FMLP (1×10^{-7} mol/L), ZAS, or HBSS were added and incubation continued for another 15 minutes. The cell suspension was then added dropwise, while stirring, to 5 mL of ice-cold 5% glutaraldehyde in PBS. After fixation of 4°C for at least four hours, the cells were centrifuged, resuspended in 0.1 mL distilled water, examined by phase-contrast microscopy, and classified by shape. A minimum of 200 cells were examined.

To study the ability of the virus to induce shape change directly, virus or AF was added to PMN in HBSS, PMN in autologous plasma, or to whole-blood leukocyte preparations in HBSS. After incubation at room temperature for 30 minutes, the cells were fixed as above. Shape change in whole blood was tested by adding 50 μ L vol of virus, AF, or FMLP (1×10^{-7} mol/L) to 500 μ L of whole

blood in EDTA (0.016 mol/L) and incubating at room temperature for 30 minutes. The buffy coat was then removed and processed as above.

Serum antibody titrations. Sera from PMN donors were tested for antibody to the virus used in these experiments by hemagglutination-inhibition assay.¹⁶

Statistical evaluation. Significance was determined by the Student's *t* test for paired data.

RESULTS

Effect on migration under agarose. Treatment with influenza virus reduced PMN chemotactic responses to FMLP and to ZAS in the migration under agarose assay (Table 1). Inhibition occurred at virus concentrations of 100 and 10 HAU:10⁶ PMN, with greater inhibition at the higher concentration. Random migration was also reduced at this virus concentration. No inhibition of migration occurred in a single experiment with virus concentration of 1 HAU:10⁶ PMN (data not shown). Migration inhibition was greater with 1×10^{-7} mol/L FMLP, a suboptimal concentration for chemotactic activity, than with 5×10^{-7} mol/L FMLP.

Viability of the virus-treated cells was $\geq 98\%$ by trypan blue exclusion. However, virus-treated cells tended to aggregate when concentrated to 5×10^7 cells/mL for testing in this assay.

Effect on migration through 100 μ m cellulose nitrate filters. To eliminate the problem of PMN aggregation, studies were performed using filter assays that employed

Table 2. Effect of Influenza Virus on PMN Migration Through 100 μ m Cellulose Nitrate Filters: PMN/mm²

Incubation Time	Chemoattractant	Migration (PMN/mm ²)*		% Inhibition*
		Virus-Treated PMN	Control PMN	
1 h	FMLP 1 × 10 ⁻⁸ mol/L	105 ± 73	186 ± 118†	46 ± 8
	FMLP 1 × 10 ⁻⁷ mol/L	88 ± 58	133 ± 73‡	38 ± 9
	FMLP 1 × 10 ⁻⁶ mol/L	0	0	
	None	0	0	
2½ h	FMLP 1 × 10 ⁻⁸ mol/L	91 ± 55	165 ± 58‡	48 ± 19
	FMLP 1 × 10 ⁻⁷ mol/L	37 ± 17	76 ± 38†	49 ± 12
	FMLP 1 × 10 ⁻⁶ mol/L	0	0	
	None	0	0	

*Mean ± SD of values from four separate experiments with PMN from different donors. Migration at one hour was measured at a filter depth of 60 μ m. Migration at 2½ hours was measured at the bottom of the filter. Virus concentration was 100 HAU:10⁶ PMN.

†Difference between virus-treated and control PMN significant at $P < 0.05$.

‡Difference between virus-treated and control PMN significant at $P < 0.02$.

Table 3. Effect of Influenza Virus on PMN Migration Through 100 μ m Cellulose Nitrate Filters: Leading Front

Chemoattractant	Migration (μ m)*		% Inhibition*
	Virus-Treated PMN	Control PMN	
FMLP 1×10^{-8} mol/L	88.8 $\pm$ 7.8	90.4 $\pm$ 5.2	8 $\pm$ 3
FMLP 1×10^{-7} mol/L	72.4 $\pm$ 9.2	78.2 $\pm$ 10.6†	
FMLP 1×10^{-6} mol/L	37.0 $\pm$ 8.5	36.2 $\pm$ 2.3	
None	40.4 $\pm$ 7.2	42.4 $\pm$ 5.8	

*Mean $\pm$ SD of values from four separate experiments with PMN from different donors. Incubation time was one hour. Virus concentration was 100 HAU:10⁶ PMN.

†Difference between virus-treated and control PMN significant at $P < 0.05$.

more dilute PMN suspension (1×10^6 /mL). There was no significant aggregation at this cell concentration, with >90% of the PMN remaining free in suspension.

The virus concentration used was 100 HAU:10⁶ PMN, and migration was measured by two methods: (1) the number of PMN migrating a given distance and (2) the position of the leading front. There was significant reduction in the number of PMN migrating 60 μ m at one hour or completely through the filter at 2½ hours (Table 2). At one hour the position of the leading front was slightly reduced with FMLP 1×10^{-7} mol/L only (Table 3). By 2½ hours the leading front had reached the undersurface of the filter with both virus-treated and control PMN.

It was noted that with these filters maximum migration occurred at FMLP 1×10^{-8} mol/L, and no chemotaxis occurred at FMLP 1×10^{-6} mol/L.

Effect on migration through 10 μ m polycarbonate filters. For the 10- μ m polycarbonate filters, MEMg was replaced by HBSS in the chambers, since migration was found to be more consistent in the absence of protein. Preliminary experiments with virus concentrations of 100 HAU:10⁶ PMN did not show any inhibition; therefore higher virus concentrations were tested. In three separate experiments using PMN from different donors, no inhibition of migration could be detected, even at virus concentrations three to 20 times greater than had been used in the previous assays (Table 4).

With these filters, maximum migration occurred at FMLP 1×10^{-6} mol/L, in contrast to the results obtained with the 100- μ m cellulose nitrate filters.

Effect on bipolar shape change. PMN treated with virus at concentrations of 320 and 2,048 HAU:10⁶ PMN showed normal percentage bipolar shape change in response to

FMLP (40.7 $\pm$ 11.1 v control 41.3 $\pm$ 9.9) and to ZAS (27.0 $\pm$ 10.6 v control 31.2 $\pm$ 10.3). Similar results were obtained at room temperature and at 37°C, with the exception of increased spontaneous shape change in unstimulated PMN at 37°C.

Influenza virus as a chemotactic agent. Influenza virus did not act as a chemotactic agent when tested in the 10- μ m polycarbonate filter assay. PMN migration was 282 $\pm$ 176 cells/mm² with influenza virus and 241 $\pm$ 195 cells/mm² with control AF.

Influenza virus induced PMN shape change when added to whole blood but not when added to purified PMN, PMN in autologous plasma, or whole blood leukocyte preparations (Table 5). In addition, plasma from virus-treated whole blood did not induce shape change in purified autologous PMN.

Antibody titers of PMN donors. Hemagglutination inhibition titers of sera from PMN donors ranged from 1:40 to 1:320. There was no correlation between antibody titer and effect of virus on PMN function.

DISCUSSION

Previous studies demonstrating in vitro inhibition of chemotaxis by influenza virus have used cellulose nitrate filter⁸⁻¹⁰ or migration under agarose^{6,7} assays. The results of our studies indicate that exposure of PMN to influenza virus in vitro did not interfere with the early stages of chemotaxis, such as the ability to recognize a chemoattractant and undergo bipolar shape change and to deform and migrate the short distance through the 10- μ m polycarbonate filter. On the other hand, there was reduction of migration when movement occurred over a greater distance, such as through

Table 4. Effect of Influenza Virus on PMN Migration Through 10- μ m Polycarbonate Filter

Virus per 10 ⁶ PMN	Chemoattractant	Migration (PMN/mm ²)*	
		Virus-Treated PMN	Control PMN
320 HAU	FMLP 1×10^{-8} mol/L	540 $\pm$ 120	476 $\pm$ 188
	FMLP 1×10^{-7} mol/L	1236 $\pm$ 220	1136 $\pm$ 232
	FMLP 1×10^{-6} mol/L	1784 $\pm$ 320	1724 $\pm$ 336
	None	216 $\pm$ 76	220 $\pm$ 68
2048 HAU	FMLP 1×10^{-8} mol/L	608 $\pm$ 436	508 $\pm$ 308
	FMLP 1×10^{-7} mol/L	1012 $\pm$ 356	992 $\pm$ 364
	FMLP 1×10^{-6} mol/L	1652 $\pm$ 244	1716 $\pm$ 332
	None	400 $\pm$ 260	332 $\pm$ 124

*Mean $\pm$ SD of values from three separate experiments with PMN from different donors. No significant differences between virus-treated and control PMN.

Table 5. Stimulation of PMN Bipolar Shape Change by Influenza Virus

Cells Tested	% Bipolar PMN*	
	Virus	Control AF
Whole blood	24.0 ± 4.6	4.7 ± 3.8†
Purified PMN	9.3 ± 5.4	8.4 ± 4.0
Purified PMN in autologous plasma	12.3 ± 4.2	11.6 ± 5.9
Whole-blood leukocyte preparation	5.5 ± 5.1	5.0 ± 5.9

*Mean ± SD of values from three to five experiments performed with blood from different donors.

†Difference between virus and control AF significant at $P < 0.01$ by paired *t* test.

the 100- μ m cellulose nitrate filter or under agarose, in agreement with previous reports.

Interpretation of results obtained in the agarose assay was confounded by virus-induced PMN aggregation. Aggregation of PMN by influenza virus was first reported in 1950 with guinea pig cells,²¹ and aggregation of human PMN by influenza A³ or B⁹ virus has been noted by others. The role this plays in limiting PMN migration in the under-agarose assay is not known. If migration inhibition were due only to aggregation, one would expect virus concentration to be the limiting factor determining the degree of inhibition, independent of the chemotactic stimulus. In fact, the degree of inhibition was less when a more potent concentration of FMLP was used.

To avoid aggregation chemotaxis was studied in a filter system, which used more dilute cell suspensions. Inhibition was observed with the 100- μ m cellulose nitrate filter but not with the 10- μ m polycarbonate filter. While both filters measure the early stages of chemotaxis, cells migrate over a much greater distance in the thick filter and may therefore be subject to modulating factors. Harvath and Leonard²² have discussed the differences in characteristics of PMN migration through these two filters. A fixed proportion of approximately 20% to 40% of the total PMN population migrates through the 10- μ m filter, with optimal response occurring at 1×10^{-6} mol/L FMLP. With the 100- μ m filters only about 15% of PMN respond, with optimal migration occurring at 5×10^{-8} mol/L FMLP and inhibition at 1×10^{-6} mol/L. These authors postulate two populations of PMN. One group responds to 1×10^{-6} mol/L FMLP and migrates the short distance through the 10- μ m filter but no further, perhaps because of chemotactic deactivation. The other group responds to 5×10^{-8} mol/L FMLP and migrates to the leading front in the thick filters. If such subpopulations do exist, our results with the 10- and 100- μ m filters may be explained by a preferential effect of influenza virus on the second group.

Influenza has been shown in previous studies to inhibit PMN adherence to plastic tissue culture plates.⁶ Since migration is dependent on optimal adherence,²³ defective adherence may limit the speed or distance of migration. The virus also inhibits degranulation of PMN specific and non-specific granules by preventing phagosome-lysosome fusion.^{6,24} Exocytosis of specific granules has been shown to play a role in modulating locomotion. These granules are normally released in small amounts during a chemotactic

response.²⁵ Virus interference with this response may limit the extent of migration. Exposure of normal neutrophils to limited concentrations of degranulating stimuli results in increased receptor binding of chemoattractants, probably as a result of translocation of specific granule membrane to the plasma membrane.²⁵ The data presented here could be explained by virus interfering with the expression of new receptors on the PMN surface. We did not examine receptor binding. However, it has previously been shown that binding of chemoattractant is increased in PMN treated with influenza virus.⁶

All PMN donors in this study had serum antibody to influenza A virus, but free antibody would have been removed in the PMN purification process. We cannot eliminate a role for PMN-bound antibody in virus-induced PMN dysfunction.

Viruses are not thought to be chemotactic, although virus-infected cells may be.²⁶ It has previously been shown that influenza virus induces PMN bipolar shape change in whole blood, suggesting that this virus may be chemotactic and therefore may interfere with migration by the process of chemotactic deactivation.⁶ In our studies the virus was not chemotactic and did not cause shape change in purified PMN, although it did cause PMN shape change in whole blood. It appeared that virus interaction with some component of whole blood induced PMN shape change. This component was not present in plasma or whole blood leukocyte preparations or in plasma from virus-treated whole blood. It may be that PMN shape change was the result of virus interaction with erythrocytes. This hypothesis could not be tested with the assay being used because distortion and aggregation of the washed erythrocytes made visualization of PMN shape change impossible. It has previously been noted that damaged erythrocytes are chemotactic.²⁷ Guinea pig erythrocytes pretreated with influenza virus have been shown to activate the alternative pathway of human complement,²⁸ but complement is unlikely to have been involved in the present studies because of the presence of EDTA.

The relevance of these in vitro findings to the inhibition of chemotaxis observed in vivo is not known, nor is it known whether or not significant PMN-virus interaction occurs in the blood stream. Maximum depression of chemotaxis has been reported at days 6 to 11 of illness in human volunteers⁴ and at days 4 to 8 in chinchillas.⁵ At this time virus concentrations in the nasopharynx have reached their peak, and antibody is beginning to appear. Direct interaction between PMN and virus would most likely occur in the respiratory tract, where high concentrations of virus are found. In vivo inhibition may be the indirect result of cellular or humoral changes induced by the disease process, as occurs in a number of other bacterial and viral infections.²⁶ It remains to be determined whether the inhibition occurring in vivo is similar to that observed here or if defects in the early stages of chemotaxis, demonstrable by shape change or migration through thin filters, also occur.

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Tyrosine Kinase and Phosphotyrosine Phosphatase Activity in Human Promyelocytic Leukemia Cells and Human Polymorphonuclear Leukocytes

By Andrew S. Kraft and Roger L. Berkow

Although an increase in protein phosphorylation on tyrosine was first noted as a result of cell transformation or the application of growth factors to cells, recent reports have shown high levels of tyrosine kinases in nondividing tissues. For that reason, we have investigated whether normal human polymorphonuclear leukocytes (PMN) contain tyrosine kinase and phosphatase activity. Using a copolymer of glutamine: tyrosine as a substrate for the phosphotransferase reaction, we have demonstrated that PMN contain a cytosolic tyrosine kinase activity that elutes as a single peak from Sephacryl S-200 chromatography and has a molecular weight of 70 kilodaltons. Human promyelocytic leukemia cells (HL-60), contain a similar activity (as demonstrated by column chromatography), with only 25% of the activity found in PMN. This cytosolic tyrosine kinase can phosphorylate angiotensin II and a fragment of the *src*

protein containing tyrosine 416, which suggests a similar substrate specificity to other tyrosine-phosphorylating protein kinases. In addition, we have demonstrated that PMN have double the amount of phosphotyrosine phosphatase (PTPase) activity of that found in HL-60 cells. This enzyme has a K_m of 0.932 mmol/L and a V_{max} of 0.355 μ mol inorganic phosphate released/mg protein/min, which is similar to other cellular PTPase. Activation of PMN with f-Met-Leu-Phe and phorbol esters causes a slight but statistically significant drop in PMN PTPase activity. These results suggest that terminally differentiated myeloid cells have high tyrosine kinase and phosphatase activity, which may play a role in stimulus response coupling in the mature PMN.

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PHOSPHORYLATION and dephosphorylation of specific proteins have been implicated in the initiation of myelomonocytic differentiation and polymorphonuclear leukocyte (PMN) activation.¹⁻⁷ Recent findings demonstrating that the receptors for many growth factors are tyrosine-phosphorylating protein kinases has led to the concept that phosphorylation and dephosphorylation involving tyrosine residues may be of importance in regulating cellular events.⁸⁻¹⁰ This is further supported by the observation that the protein products of transforming viral oncogenes are tyrosine kinases.^{8,9,11} Many of these transforming genes have a normal cellular homologue.¹² The finding of phosphotyrosine-specific phosphatases in a wide variety of cells¹³⁻²⁹ and the observation that inhibitors of these phosphatases must be included when isolating oncogene-associated tyrosine kinases^{9,30-32} further suggest that phosphorylation and dephosphorylation of tyrosine may regulate cellular events.

Recent reports have demonstrated that normal hematopoietic cells contain oncogene-coded tyrosine kinases.^{31,33-35} Platelets have been shown to contain *c-src*, the cellular homologue of the Rous sarcoma virus-transforming protein.³¹ In addition, *c-src* has been shown to increase in HL-60 cells induced to mature to granulocytes or macrophages.³⁶ Furthermore, the platelet-derived growth factor (PDGF), whose receptor is known to have tyrosine kinase activity,^{8,9,37}

has been shown to activate PMN to undergo chemotaxis, to produce superoxide, and to release granule contents.³⁸ These data suggest that tyrosine phosphorylation and dephosphorylation may play a specific role in terminally differentiated myeloid cells.

We demonstrate that both HL-60 cells and normal human PMN contain a 70-kilodalton (kDa) cytosolic tyrosine kinase activity and that neutrophils have fourfold greater activity than is found in the HL-60 cells. In addition, PMN contain a membrane-associated phosphotyrosine phosphatase activity that has twofold greater activity than is seen in the HL-60 cells. Activation of PMN with either f-Met-Leu-Phe (fMLP) or phorbol esters induces a significant drop in tyrosine phosphatase activity. Our data suggest that tyrosine kinase and phosphatase activity increases during myelocytic differentiation and that these enzymes may play a role in stimulus response coupling in the mature PMN.

MATERIALS AND METHODS

Materials. Copolymer-containing tyrosine:glutamic acid (20:80) and angiotensin II were purchased from Sigma Chemical Co, St Louis; Filtron-X scintillation fluor was purchased from National Diagnostics, Manville, NJ; all *trans*-retinoic acid was purchased from Eastman Kodak, Rochester, NY; and [γ -³²P] adenosine triphosphate (ATP) was purchased from Amersham Corp, Arlington Heights, IL. Phorbol myristate acetate (PMA) was supplied by PL Biochemical Corp, Milwaukee, and the peptide RR-*src* was supplied by Peninsula Labs, Belmont, CA.

Phosphotyrosine, phosphoserine, phosphothreonine, EDTA, sodium orthovanadate, HEPES, *p*-nitrophenylphosphate, and fMLP were obtained from Sigma. All other chemicals were reagent grade.

Cell culture. HL-60 cells were provided by Dr J. Fontana (West Virginia University, Morgantown) and were early passage; KG-1 cells were supplied by Dr P. Koeffler (UCLA) and K-562 by Dr L. Bertoli (University of Alabama at Birmingham). All cell lines were grown at 37°C in RPMI 1640 media (GIBCO, Grand Island, NY) containing 10% fetal calf serum, antibiotics (50 U/mL of penicillin and 50 μ g/mL of streptomycin), and 4 mmol/L glutamine in a 5% CO₂ humidified atmosphere. PMN were isolated from normal human donors by counterflow centrifugal elutriation according to our previously described method.³⁹

After isolation, PMN were suspended in either 0.1 mol/L sodium

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acetate buffer, pH 6.0, with 1 mmol/L EDTA and 0.2% (vol/vol) Triton X-100 (buffer A) or a buffer containing 145 mmol/L NaCl, 4.9 mmol/L KCl, 1 mmol/L CaCl_2 , 1 mmol/L MgSO_4 , 16 mmol/L HEPES, and 1 mg/mL glucose, pH 7.4 (buffer B).

Protein kinase assays. Cells were washed three times with Dulbecco's phosphate-buffered saline. They were dounce homogenized 30 strokes in a buffer containing 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 0.5 mmol/L ethylene glycol tetraacetic acid (EGTA), 0.33 mol/L sucrose, and 2 mmol/L phenylmethylsulfonyl fluoride. They were then centrifuged at 100,000 g for one hour and the supernatant used as a source of cytosolic enzyme. Tyrosine kinase activity was measured by using copolymer-containing tyrosine:glutamic acid (20:80) as a phosphotransferase acceptor. The reaction was initiated by the addition of 50 μL of enzyme preparation to a 250- μL reaction mixture containing 20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L Mg acetate, 2.5 mmol/L Mn chloride, 100 $\mu\text{mol/L}$ [$\gamma\text{-}^{32}\text{P}$] ATP (60 cpm/pmol), and 250 μg copolymer. After incubation for five minutes at 30°C, the reaction was terminated with 2 mL of 20% trichloroacetic acid, and the precipitate was collected by filtering over a Millipore (Freehold, NJ) filter (0.45 μm). Filters are washed with 10 mL of 5% trichloroacetic acid, dissolved in Filtron-X scintillation fluor, and counted for radioactivity. Enzyme activity was linear up to 15 minutes. Triplicate determinations agreed to within $\pm 10\%$. To determine specific tyrosine phosphotransferase activity, the amount of ^{32}P incorporation in the absence of copolymer was subtracted from the amount of ^{32}P incorporated with copolymer present.

Phosphorylation of peptide substrates. The phosphorylation reaction was done, as described previously,⁴⁰ in a 20- μL volume containing 20 μg of RR-src or angiotensin II, 20 mmol/L Tris-HCl, pH 7.5, 100 $\mu\text{mol/L}$ vandate, 10 mmol/L MgCl_2 , 2.5 mmol/L MnCl_2 , 10 $\mu\text{mol/L}$ ATP, and 10 μL of partially purified tyrosine kinase. The reaction was started by the addition of 2 μL of [$\gamma\text{-}^{32}\text{P}$] ATP and incubated at 30°C for ten minutes. The reaction was terminated by the addition of 20 μL of 60% acetic acid and spotted onto Whatman P-81 paper. The filter squares were then washed in 30% acetic acid for 45 minutes followed by a wash in acetone for five minutes. The filter squares were counted in Aquasol-2 (New England Nuclear, Boston).

Enzyme assays. Alkaline phosphatase activity was determined by the method of DeChatelet and Cooper using *p*-nitrophenyl phosphophosphate as the substrate and 2-amino-2-methyl propanol, pH 10.0, containing 1 mmol/L MgCl_2 as the buffer.⁴¹ Phosphoprotein phosphatase activity was determined as follows according to the method of Leis and Kaplan.²¹ PMN at $2 \times 10^7/\text{mL}$ in buffer A were sonicated to disrupt the cells and centrifuged at 400 g to remove unbroken cells and cell nuclei. The supernatant was then used for the assay (400-g supernatant). To a total volume of 0.5 mL is added 75 μL of 400-g supernatant containing approximately 75 μg of protein and 10 mmol/L phosphotyrosine in buffer A. The reaction was allowed to proceed for 15 minutes at 37°C and was stopped by the addition of 150 μL of 100% (wt/vol) trichloroacetic acid. The tube was then cooled on ice for 60 minutes. The precipitate was removed by centrifugation at 10,000 g and the supernatant assayed for inorganic phosphate (Pi) according to the method of Fiske and Subbarow.⁴²

Protein was determined by the method of Lowry et al⁴³ or the method of Bradford.⁴⁴

RESULTS

Tyrosine kinase activity of HL-60 cells and circulating PMN. Using a copolymer containing tyrosine and glutamic acid as a phosphotransferase acceptor, we partially purified a tyrosine kinase from HL-60 cells. This enzyme can be measured in crude homogenates of HL-60 cells and was further purified by elution from a diethylaminoethyl (DEAE)-cellulose column with 0.05 mol/L NaCl (Fig 1A). After dialysis, this tyrosine kinase activity was further purified by phosphocellulose chromatography (Fig 1B). This enzyme, which eluted as a single peak from both of these columns, was active in the presence of manganese or magnesium ions, with the former stimulating greater phosphorylation of copolymer (Fig 2). In comparison, the addition of varying concentrations of calcium, cobalt, or zinc had no effect on phosphotransferase activity. Also, the addition of

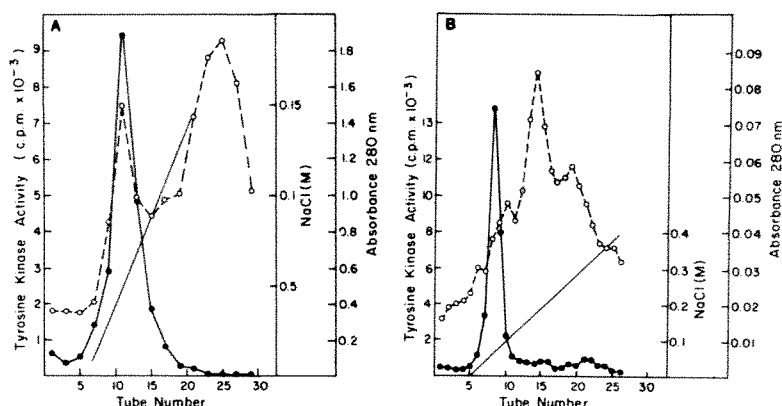


Fig 1. Partial purification of tyrosine kinase from HL-60 cells. (A) DEAE-cellulose chromatography. The 100,000-g supernatant prepared from 10^9 HL-60 cells was applied to a DEAE-cellulose column (0.9×20 cm) equilibrated with buffer C (20 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride). After sample addition, the column was washed extensively with buffer C and then eluted with a linear gradient of 70 mL of buffer C plus 70 mL of buffer A containing 0.2 mol/L NaCl. Fractions of 2 mL were collected. Aliquots (50 μL) were assayed for tyrosine kinase activity ($\bullet$ — $\bullet$). Protein kinase activity is expressed as counts per minute of ^{32}P incorporated per five minutes per 50- μL aliquot. NaCl concentration is denoted by (—). Absorbance at 280 nm is denoted by (O—O). (B) Phosphocellulose chromatography. Fractions containing tyrosine kinase activity eluted from DEAE-cellulose were dialyzed against three changes of buffer C. They were then loaded onto a phosphocellulose column (0.9×11 cm) and washed extensively to remove unbound protein. The column was then eluted with a 60-mL 0 to 0.4 mol/L salt gradient in buffer A. Two-milliliter fractions were collected and assayed as before. The NaCl concentration is denoted by (—). Absorbance at 280 nm is denoted by (O—O).

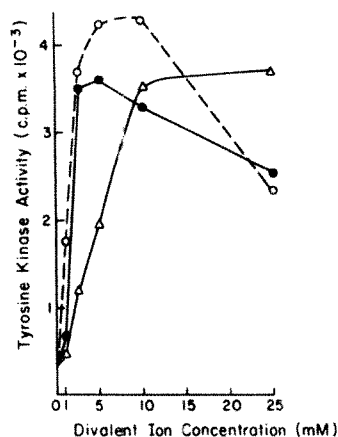


Fig 2. The effect of increasing divalent ion concentration on tyrosine kinase activity from HL-60 cells and PMN. The activity of tyrosine kinase from HL-60 cells partially purified as in Fig 1 was determined with increasing concentrations of manganese (O---O) or magnesium (Δ---Δ). A similar experiment was done at a later time with partially purified tyrosine kinase from PMN in which the effect of varying concentrations of manganese (●---●) was assayed.

phosphatidylserine (80 $\mu\text{g}/\text{mL}$), PMA (1 $\mu\text{mol}/\text{L}$) alone or in combination with calcium (1 mmol/L) and phosphatidylserine (80 $\mu\text{g}/\text{mL}$), epidermal growth factor (0.3 to 3 $\mu\text{mol}/\text{L}$), or insulin (0.2 μM) did not stimulate this tyrosine kinase. Acidic and basic histones were poor substrates for this kinase, whereas a peptide-containing tyrosine 416 of the *src* protein (RR-*src*) or angiotensin II (Table 1) were both phosphorylated.

Because HL-60 cells are a promyelocytic leukemia cell line blocked at an early stage in differentiation,^{1,3} we were interested in whether tyrosine kinase activity would be altered as the cell matures. To examine this possibility, we assayed tyrosine kinase activity in HL-60 cells induced to differentiate toward granulocytes with retinoic acid (1 $\mu\text{mol}/\text{L}$) or toward macrophages with PMA (10 nmol/L). For comparison we assessed the activity of this enzyme in mature granulocytes isolated from human peripheral blood as well as in the KG-1 cell line, a myeloid cell line less mature than HL-60 cells, and the K562 cell line, which has properties of both myeloid and erythroid progenitors.

The tyrosine kinase activity was measured on equivalent amounts of protein from the 100,000-g supernates of these cell lines. Table 2 shows that human PMNs have approxi-

Table 2. Relative Tyrosine Kinase Activity in PMNs and Leukemic Cell Lines When Compared With HL-60 Cells

Cell Line	Relative Tyrosine Kinase Activity (%)
HL-60 treated with retinoic acid (1.0 $\mu\text{mol}/\text{L}$) $\times$ 5 d	133 $\pm$ 21
HL-60 treated with PMA (10 nmol/L) $\times$ 5 d	68 $\pm$ 16
KG-1	87 $\pm$ 11
K562	34 $\pm$ 14
PMN	460 $\pm$ 56

Equivalent amounts of protein from a 100,000-g supernatant were assayed for tyrosine kinase activity as described in Materials and Methods. Control HL-60 supernatants incorporated approximately 100 cpm/ μg protein per 5 min into copolymer. Results are the means of triplicate determinations $\pm$ SD.

mately fourfold greater activity than was found in HL-60 cells. Differentiation of the HL-60 cells to granulocyte-like cells caused small increases in enzyme activity, whereas differentiation with PMA to macrophage-like cells caused a slight decrease in activity. Both KG-1 and K562 cell lines had less cytosolic tyrosine kinase activity than that found in the HL-60 cells.

Since HL-60 cells and mature PMN contain cytosolic tyrosine kinase activity, we examined whether this activity might be determined by similar if not identical proteins. The tyrosine kinase activity in both PMN and HL-60 cytosols elutes from a DEAE-cellulose column at 0.05 mol/L NaCl. In addition, partially purified tyrosine kinase activity from the cytosol of PMN has a similar dependence on manganese (Fig 2). By using a Sephacryl S-200 molecular sieve column both the HL-60 and PMN cytosolic tyrosine kinases were found to have a molecular weight of approximately 70 kDa (Fig 3).

Phosphotyrosine phosphatase activity of circulating PMN and HL-60 cells. Because PMN demonstrated a high cytosolic tyrosine kinase activity, we hypothesized that these cells should contain phosphotyrosine phosphatase activity. We therefore determined phosphotyrosine phosphatase activity in a 400-g supernatant of human PMN obtained as described in Materials and Methods. Analysis of this enzyme activity revealed 0.399 ± 0.014 μmol Pi released from the phosphotyrosine substrate per milligram of cell protein per minute (mean $\pm$ SE, $n = 17$). This activity was linear with respect to both the amount of cellular protein assayed, from 10 to 150 μg , and with respect to the time of incubation of the protein with substrate, 15 to 30 minutes. Under these same conditions and with 10 mmol/L phosphoserine and phosphothreonine as the substrates, the PMN 400-g supernatant demonstrated 0.012 ± 0.001 and 0.045 ± 0.001 μmol Pi released from the substrate/mg/min (mean $\pm$ SE, $n = 3$), respectively. This indicated that by using phosphoamino acids the phosphotyrosine phosphatase activity was greater in the 400-g supernatant than phosphatases for phosphoserine or phosphothreonine. The kinetics of phosphotyrosine phosphatase activity in the PMN 400-g supernatant was then determined by Lineweaver-Burke analysis. A double reciprocal plot (Fig 4) demonstrated that this enzyme

Table 1. Phosphorylation of Peptides by Partially Purified Tyrosine Kinase From HL-60 Cells

Additions	Tyrosine Kinase Activity (cpm/20 μg Protein/10 min)
RR- <i>src</i>	6,206
Angiotensin II	2,777
None	1,139

Tyrosine kinase is purified by DEAE-cellulose chromatography and added to a reaction mixture containing RR-*src* or angiotensin II. The reaction was carried out as described in Materials and Methods and stopped by the addition of 60% acetic acid. The data presented are the means of duplicate experiments that vary $\pm 15\%$.

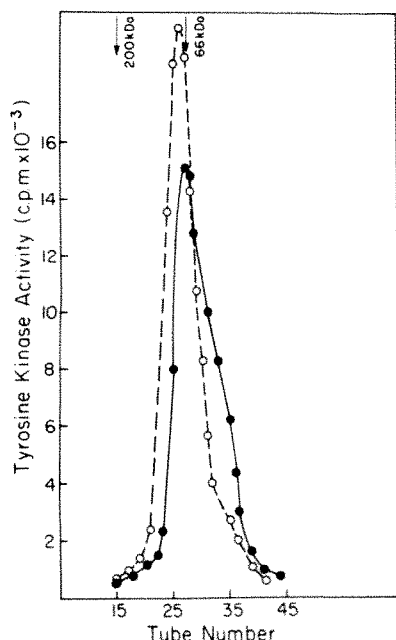


Fig 3. Sephacryl S-200 filtration chromatography of HL-60 cells and PMN. Each cell type was homogenized in 2 mL of buffer as described in Results. One milliliter was passed through a Sephacryl S-200 column (1.5 × 30 cm) equilibrated with 20 mol/L Tris, pH 7.5, and eluted with the same buffer. Fractions (1 mL) were collected, and 50- μ L aliquots of each fraction were assayed for tyrosine kinase activity as described. Protein kinase activity is expressed as in the legend to Fig 1. HL-60 tyrosine kinase activity is denoted by (○---○) and PMN by (●—●). Arrows denote the standards, 66 kDa denotes bovine serum albumin and at 200 kDa, β -amylase.

had an apparent K_m of 0.932 mmol/L and a V_{max} of 0.355 μ mol Pi released/mg protein/min.

To assess the location of the phosphotyrosine phosphatase activity the PMN were suspended in 0.1 mol/L sodium acetate buffer with 1 mmol/L EDTA and sonicated as

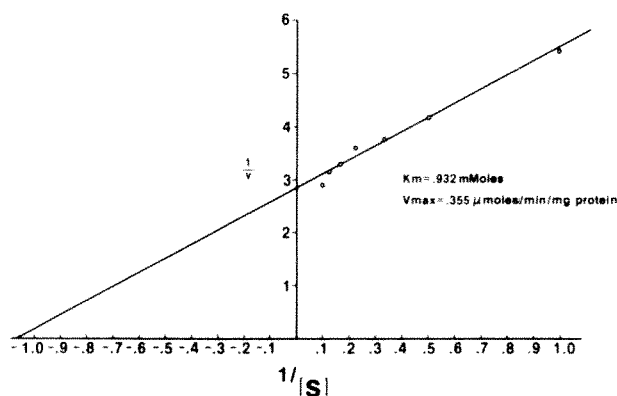


Fig 4. Double reciprocal plot of phosphotyrosine phosphatase activity in the PMN 400-g supernatant. Fifty to 75 μ g of the PMN 400-g supernatant was incubated at 37°C for 15 minutes with varying concentrations of phosphotyrosine as substrate in 0.1 mol/L sodium acetate buffer, pH 6.0, with 0.2% (vol/vol) Triton X-100 and 1 mmol/L EDTA. The results are a representative experiment of at least three different experiments that varied by less than 5%.

described. When the 400-g supernatant was centrifuged at 100,000 g at 4°C for 60 minutes, the 100,000-g pellet contained phosphotyrosine phosphatase activity equivalent to 0.315 ± 0.013 μ mol of substrate hydrolyzed/mg protein/min. This represented 87% of the total cellular activity. The 100,000-g supernatant contained 0.046 ± 0.007 μ mol/mg/min and represented 13% of the total activity (mean $\pm$ SE, $n = 5$).

We next determined the optimum pH of the phosphotyrosine phosphatase in the PMN 400-g supernatant. Figure 5 shows that this enzyme demonstrated a broad range of activity, with optimum hydrolysis of phosphotyrosine occurring at pH 7.0.

In membrane-associated fractions, PMN are known to contain a large amount of alkaline phosphatase activity. This enzyme activity is dependent on magnesium ions and is inhibited by chelators of divalent cations.⁴⁵ It was important therefore to distinguish our phosphotyrosine phosphatase activity from PMN alkaline phosphatase. Alkaline phosphatase activity assessed at pH 10.0 using *p*-nitrophenyl phosphate as the substrate demonstrated a requirement for magnesium ions and a distinct dose-dependent inhibition of activity by 0 to 10 mmol/L EDTA (36% of control at 2 mmol/L EDTA in the presence of 1 mmol/L $MgCl_2$). Figure 6 demonstrates that EDTA over a concentration range of 0 to 10 mmol/L had no effect on the hydrolysis of phosphotyrosine by the PMN 400-g supernatant. Similarly, $CaCl_2$ and $MgCl_2$ (0 to 10 mmol/L) had no effect on phosphotyrosine phosphatase activity (data not shown). In comparison a dose-dependent inhibition of hydrolysis of phosphotyrosine occurred when sodium orthovanadate, $ZnCl_2$, or $MnCl_2$ was added to the PMN 400-g supernatant before addition to the substrate. This pattern of inhibition is similar to that seen for phosphotyrosyl protein phosphatases from other tissue sources.¹³⁻²⁹

To determine whether phosphotyrosine phosphatase activ-

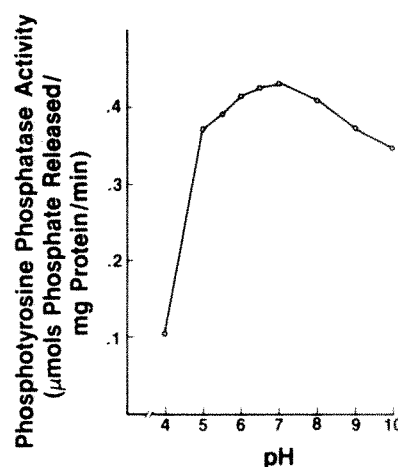


Fig 5. Effect of pH on phosphotyrosine phosphatase activity. The activity was determined in 0.1 mol/L acetate buffer adjusted to the given pH by using 10 mmol/L phosphotyrosine as substrate under the conditions noted in Fig 4. The results are expressed as micromoles of Pi released from the substrate per milligram of protein per minute. This curve represents the means of three separate experiments performed in duplicate.

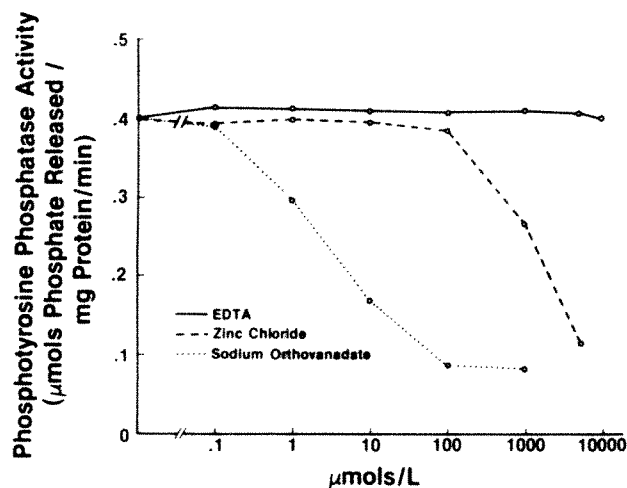


Fig 6. Effect of phosphatase inhibitors on PMN phosphotyrosine phosphatase activity. PMN 400-g supernatant was incubated with the stated concentration of sodium orthovanadate, $ZnCl_2$, or EDTA before assessment of phosphotyrosine phosphatase activity as described in Results. The results are means of three to five separate experiments and are expressed as a percentage of the phosphotyrosine phosphatase activity seen in the absence of the test substance.

ity increases during myeloid differentiation, we next evaluated undifferentiated HL-60 cells for phosphotyrosine phosphatase activity. A 400-g supernatant prepared from HL-60 cells demonstrated 0.211 ± 0.05 μ mol Pi hydrolyzed from phosphotyrosine per milligram of cell protein. This was 52.8% of the amount of phosphotyrosine phosphatase activity found in the PMN.

To help understand the role of the phosphotyrosine phosphatase in the PMN we examined the activity of this enzyme in PMN suspended in buffer B and stimulated with varied concentrations of PMA, fMLP, or fMLP plus 5 μ g/mL of cytochalasin B (CB). Figure 7 demonstrates that within five minutes of incubation with PMA or fMLP plus cytochalasin B, a dose-dependent reduction in phosphotyrosine phosphatase activity was seen. At the highest concentration of PMA or fMLP tested, phosphotyrosine phosphatase activity of $71.6\% \pm 6.6\%$ and $75.8\% \pm 8.5\%$ of control value, respectively, was found (mean $\pm$ SD, $n = 5$). In contrast to the PMN granule constituents lysozyme, myeloperoxidase, and vitamin B_{12} -binding protein, which are released into the incubation media upon PMN activity with PMA or fMLP, no phosphotyrosine phosphatase activity was released upon PMN stimulation by these agents (data not shown).

DISCUSSION

In this communication, we have presented data demonstrating that myeloid leukemia cell lines and mature circulating neutrophils contain both cytosolic tyrosine protein kinase activity and membrane-associated phosphotyrosine phosphatase activity. The cytosolic tyrosine kinase activity from both the PMN and HL-60 cells has a similar molecular weight of 70 kDa. The elution profile from DEAE-cellulose and sensitivity to Mg^{+2} and Mn^{+2} are also similar, which suggests that

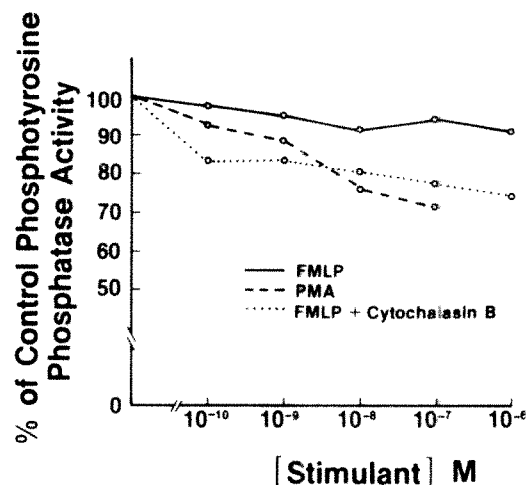


Fig 7. Effect of PMN stimulation on phosphotyrosine phosphatase activity. PMN at 2×10^7 /mL in buffer B were incubated at 37°C for five minutes with varying concentrations of PMA, fMLP, or fMLP in the presence of 5 μ g/mL of cytochalasin B. The reaction was stopped by placing the cells at 4°C . The cells were then centrifuged at 400 g (the resultant cell-free supernatant is the poststimulated supernatant). The cells were resuspended in buffer A and sonicated as described and a 400-g supernatant obtained. The phosphotyrosine phosphatase and protein content of the 400-g supernatant was then determined. Similarly, 150 μ L of the stimulation incubation media was assessed for phosphotyrosine phosphatase activity.

these may be related if not identical enzymes. Further characterization will be needed, however, to establish their identity. Although PMN have approximately fourfold more cytosolic tyrosine kinase activity than the uninduced HL-60 cells, treatment of the HL-60 cells with retinoic acid to induce a granulocyte phenotype resulted in only a slight elevation of activity. Since we assayed the effects of PMA and retinoic acid five days after treatment, it is possible that we did not observe earlier changes in tyrosine kinase activity induced by these agents. Of interest is the ability of the 70-kDa cytosolic tyrosine to phosphorylate a peptide-containing tyrosine 416 of the *src* protein. Activation of other tyrosine kinases, for example, the PDGF receptor, has been shown to modulate membrane-associated *src* tyrosine kinase activity in fibroblasts.³⁷ Our result might imply a possible role for the 70-kDa cytosolic tyrosine kinases in modulation of membrane-bound tyrosine-phosphorylating protein kinases. Furthermore, both platelets and rat liver contain a cytosolic tyrosine kinase activity with a similar molecular weight to that found in the present study, thereby suggesting that this activity is not specific to myeloid cells or myeloid differentiation.^{46,47}

Numerous substrates have been used to evaluate phosphotyrosine phosphatase activity including phosphorylated histone, angiotensin, fragments of the *src* oncogene, casein, albumin, and myosin light chains.¹³⁻³² Although the physiological substrates of this enzyme are not known, others have demonstrated that acidic protein substrates are dephosphorylated by phosphotyrosine phosphatase^{16,45} whereas alkaline substrates are dephosphorylated by membrane-associated alkaline phosphatase.^{16,45} We have used phospho-

tyrosine as the substrate in the present study. Although this is not a peptide or protein substrate, our data and that of others^{21,22} suggest that this phosphorylated amino acid may be used to measure specific phosphotyrosine phosphatase activity.

The data presented demonstrate that PMN and cells contain phosphotyrosine phosphatase activity that is localized to the cell particulate fraction. This enzyme has activity over a broad pH range (Fig 5), which indicates that the PMN may contain several species of phosphotyrosine phosphatase, as demonstrated in rat spleen and bovine brain.²⁸ Both the K_m (0.932 mmol/L) of this enzyme and its inhibition by zinc, vanadate, and manganese ions are similar to that described for phosphotyrosine phosphatases isolated from fibroblasts, placenta, A431 cells, astrocytoma cells, and *Drosophila*.¹³⁻³² Unlike PMN alkaline phosphatase,⁴⁵ PMN phosphotyrosine phosphatase is not dependent on magnesium ions, nor is it inhibited by EDTA, thereby suggesting that these phosphatases are not the same enzyme. However, purification of both enzymes will be necessary to demonstrate that these are totally separate activities. In addition, we have found that PMN contain approximately twofold

greater phosphotyrosine phosphatase activity than that in uninduced HL-60 cells. Our results are in accord with recently presented data demonstrating an increase in membrane-associated phosphotyrosine phosphatase activity induced in HL-60 cells by retinoic acid.⁴⁸

We have evaluated the effect of fMLP and PMA on PMN phosphotyrosine phosphatase activity. Treatment of PMN for five minutes with fMLP plus CB or PMA causes a slight drop in phosphotyrosine phosphatase activity ($P < .05$). Because no phosphotyrosine phosphatase activity is found in the incubation media after PMA activation, these data suggest that PMN phosphotyrosine phosphatase is not secreted and is probably not a granule constituent. Furthermore, in preliminary experiments (data not shown), we have demonstrated that activation of PMN with PMA or fMLP leads to increased phosphorylation of protein bands that demonstrate resistance to alkali treatment, thereby implying phosphorylation on tyrosine residues.⁴⁹ These data may indicate that ligand binding to PMN leads to phosphorylation of proteins on tyrosine residues by the activation of a tyrosine kinase and the inhibition of phosphotyrosine phosphatase activity.

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Actin Polymerization and Its Relationship to Locomotion and Chemokinetic Response in Maturing Human Promyelocytic Leukemia Cells

By William H. Meyer and Thomas H. Howard

We studied actin polymerization in the HL-60 human promyelocytic leukemia cell line during induced myeloid maturation and its relationship to the rate of locomotion (ROL). The percent G-actin (of total actin) was measured by DNAase I inhibition, F-actin was determined by fluorescence-activated cell sorter (FACS) analysis of nitrobenzoxadiazol (NBD)-phalloidin-stained cells, and ROL was measured by computer-assisted analysis of the tracks of individual cells. Uninduced HL-60 cells moved slowly ($2.3 \pm 1.0 \mu\text{m}/\text{min}$) and showed no change in ROL or in the state of actin polymerization when stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP). Nonstimulated cells induced to differentiate with dimethylformamide had no change in the degree of actin polymerization but exhibited a mean ($\bar{m}$) ROL similar to normal human polymorphonuclear leukocytes (PMN) ($8.6 \pm 1.4 \mu\text{m}/\text{min}$ [HL-60 cells] v

$7.8 \pm 1.8 \mu\text{m}/\text{min}$ [PMN]). When induced HL-60 cells were stimulated with fMLP, actin polymerization occurred. The F-actin content increased, as determined by FACS analysis of NBD-phalloidin-stained cells, and the percentage of G-actin decreased, as determined by a 24.5% decrease in DNAase I inhibitory activity. However, induced HL-60 cells stimulated with fMLP did not increase their $\bar{m}$ ROL. These studies show that, unlike normal human PMN, chemotactic peptides can cause an intracellular biochemical change that is not associated with a chemokinetic response in induced HL-60 cells. The HL-60 cell line may be a useful model to study the development of chemotactic peptide-mediated actin polymerization during myeloid cell maturation.

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THE HL-60 HUMAN promyelocytic leukemia cell line can be induced to differentiate and has been used as a model to study morphological, functional and biochemical changes during this process of maturation.¹⁻³ One such area of investigation has been the development of chemotaxis. Uninduced HL-60 cells are relatively nonmotile. During induced myeloid maturation of HL-60 cells, the number of chemotactic peptide receptors increases,^{4,5} and cells have a chemotactic response, as demonstrated by the Boyden chamber assay and agarose assay to assess motility.⁵⁻⁷ However, induced HL-60 cells differ from normal human neutrophils. A much larger proportion of normal neutrophils migrate to significant distances within micropore filters than do HL-60 cells induced with *N,N*-dimethylformamide (DMF).⁵ Under agarose, the leading front of the HL-60 cells differentiated with dimethyl sulfoxide (DMSO) for six days and when stimulated with formyl-methionyl-leucyl-phenylalanine (fMLP) is 6.9 ± 0.7 cm compared with 14.1 ± 0.7 cm observed in human polymorphonuclear leukocytes (PMN).⁷ Chemotaxis of induced HL-60 cells is also significantly less than that of PMN when pepstatin, *Escherichia coli* supernatant, or zymosan-activated serum are used as attractants.⁷

Actin is a ubiquitous protein in eukaryotic cells and has a central structural role in nonmuscle cells. In addition, actin is primarily involved in cell motility. During induced myeloid maturation of a mouse myeloid leukemia cell line (M1), increases in actin content and actin synthesis were noted. Also, G-actin showed a greater ability to polymerize, which was thought to be a phenomenon specifically related to the induction of motility and phagocytosis.⁸ We have demonstrated, using human PMN, that stimulation by fMLP (up to 10^{-8} mol/L) induces a chemokinetic response. This response coincided with an increase in the content of polymerized actin (F-actin), as determined by nitrobenzoxadiazol (NBD)-phalloidin staining, and by a decrease in the content of globular actin (G-actin), as determined by deoxyribonuclease I (DNAase I) inhibition.⁹ Because induced HL-60 cells are reported to demonstrate a chemotactic response, we investigated the quantity and polymerization of actin and the motile behavior of HL-60 cells induced toward myeloid

maturation. We reported previously that during a five-day exposure of HL-60 cells to DMF, the actin content increases twofold, thus reflecting actin synthesis.¹⁰

We report that (a) induced HL-60 cells become motile and have a rate of locomotion (ROL) similar to human PMN; (b) during induced myeloid differentiation, the G-actin percentage of total actin remains unchanged despite a twofold increase in total actin; (c) stimulation of differentiating HL-60 cells with fMLP causes actin polymerization; however, actin polymerization was unchanged by fMLP stimulation of uninduced HL-60 cells; and (d) in spite of the effect of fMLP stimulation on actin polymerization, no chemokinetic response occurred in differentiating HL-60 cells.

MATERIALS AND METHODS

Cells. HL-60 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and induced toward myeloid maturation with 120 mmol/L DMF. Cells in passage number 10-40 were used. The cells were a gift from Dr Curt Civin, who had previously obtained an early passage of Dr S.J. Collins' original line. We obtained some of this cryopreserved stock, passaged the line three to six times, and refroze the cells in 10% DMSO in liquid nitrogen. The passage number indicates the passage since thawing from our cryopreserved stock. Treated cells showed expected myeloid maturation as assessed by Wright-stained cytospin preparations and reduction of nitroblue tetrazolium as previously reported.¹⁰

Human neutrophils (PMN) were isolated from peripheral blood by dextran sedimentation and Ficoll-Hypaque separation.⁹

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Locomotion studies. Locomotion was measured by tracking cells suspended in Hanks/HEPES buffer (pH 7.15) with 2.0% albumin at 37°C. Cells were videorecorded, and tracings were digitized and analyzed with computer assistance as previously described.⁹ Cells were recorded at low magnification and resolved as large dots on the monitor.

So that extraneous debris would not be confused with cells during tracking, viable HL-60 cells were separated from debris and any dead cells by Ficoll-Hypaque gradients, which yielded >95% viable cells. This step also controls for any artifacts introduced by Ficoll separation required to obtain pure PMN populations.

DMSO, 0.1% (vol/vol), with or without fMLP was added immediately before injecting cells into the chamber.

Measurement of actin by DNAse I inhibition. G- and total actin pools were measured with the previously reported^{9,10} modified DNase I assay of Blikstad et al.¹¹ The assay conditions were as follows: DNA substrate (80 µg/mL calf thymus DNA [Sigma Chemical Co, St Louis]) reacted with 20 µL DNase I (10 mg/mL type I, chromatographically purified [Sigma]) at 30°C and 260 nm. Total actin was determined from a separate standard curve constructed after the addition of 20 µL of 1.5 mol/L guanidine HCl solution because this solution inhibits the reaction. Cells were stimulated with fMLP as in the locomotion studies.

NBD-phalloidin staining and analysis. The staining and analysis procedure has been previously reported.⁹ Cells were stained after a ten-minute incubation in DMSO (0.1%, vol/vol) with or without 10^{-8} mol/L fMLP at 37°C in Hanks/HEPES buffer. Cells were fixed, permeabilized, and stained in a single step with 0.1 mL 37% phosphate-buffered formalin containing 1.65×10^{-6} mol/L NBD phalloidin (Molecular Probes, Junction City, OR) and 100 µg lysophosphatidylcholine.

Cells were analyzed on a Becton Dickinson model IV fluorescence-activated cell sorter (FACS; Becton Dickinson, Oxnard, CA), excited at 488 nm, and read at 522 nm. Results were displayed as histograms of fluorescence and recorded on Polaroid positive prints.

RESULTS

Locomotion studies. To analyze changes in motility with the induction of myeloid maturation, we determined the mean ROL ($\bar{m}$ ROL) of HL-60 cells in 0.1% (vol/vol) DMSO alone. One hundred to 150 cells were videorecorded for ten minutes, and the ROL was determined as previously described. As shown in Fig 1, day 0 (uninduced) HL-60 cells moved slowly (ROL, 2.3 ± 1.0 µm/min). HL-60 cells induced with DMF showed an increased ROL; by day 3, the $\bar{m}$ ROL was similar to more fully matured day 5 HL-60 cells. The $\bar{m}$ ROL of day 5 cells (8.6 ± 1.4 µm/min) is similar to that of human PMN (7.8 ± 1.8 µm/min) studied under the same conditions.

Stimulation of HL-60 cells with 10^{-8} mol/L fMLP failed to increase the $\bar{m}$ ROL. This lack of chemokinetic response to fMLP was noted in uninduced HL-60 cells and for all groups of cells exposed to DMF for up to five days. Human PMN exposed to 10^{-8} mol/L fMLP under the same experimental conditions moved at a $\bar{m}$ ROL of 12.2 ± 2.2 µm/min, a 50% increase over nonstimulated cells. Induced HL-60 cells had the expected degree of morphological maturation as determined from Wright-stained cytospin preparations. In addition, HL-60 cells appeared similar to PMN in their ability to spread and deform. No attempt was made to assess adherence, and since no gradient exists in the chemokinetic assay, orientation to a gradient would not occur.

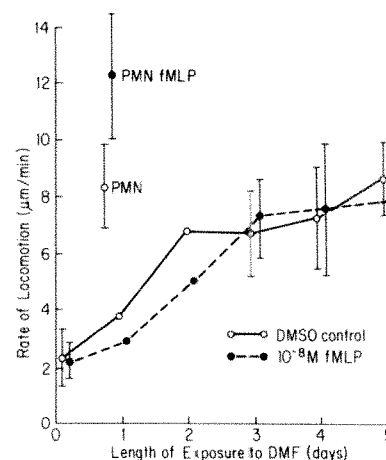


Fig 1. Mean $\pm$ SD for ROL v duration of exposure to DMF. The $\bar{m}$ ROL of HL-60 cells exposed to DMF for zero (control) through five days was determined in the presence of 10^{-8} mol/L fMLP (dissolved in 0.1% DMSO) (closed circles) or in DMSO (0.1% vol/vol) alone as a control (open circles). For days 0, 3, 4, and 5, a mean $\pm$ SD was determined from the $\bar{m}$ ROL of three experimental trials. For days 1 and 2, a single trial was done. Normal human PMN ROL (in 0.1% DMSO) is shown for comparison.

To determine whether the ROL of a subpopulation of induced HL-60 cells would increase after stimulation with fMLP, histograms were made by plotting the percentage of cells having a specific ROL during five minutes of observation. As shown in Fig 2, the distribution of locomotion rates of day 5 HL-60 cells was similar with and without stimulation by 10^{-8} mol/L fMLP. No subpopulation of cells had a chemokinetic response to fMLP. The $\bar{m}$ ROL and distribution histograms for day 5 HL-60 cells were reproducible for experimental trials done as long as 1 year apart.

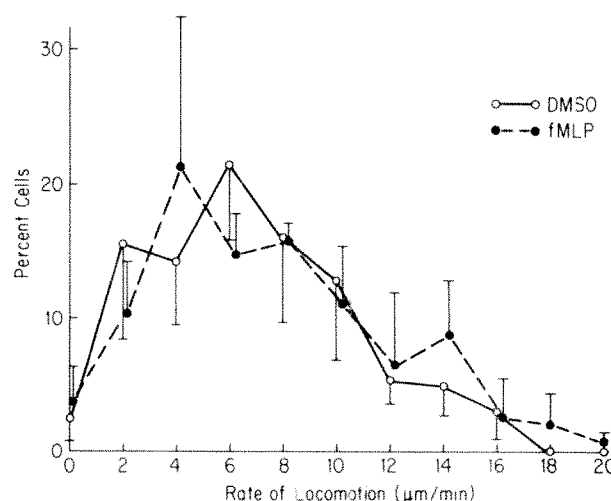


Fig 2. Histogram of ROL for HL-60 cells exposed to DMF for five days. The ROL (µm/min) was determined during a five-minute period of observation. The percentage of cells exhibiting the specified ROL is plotted by 2-µm/min subclasses. Open circles indicate control cells (0.1% DMSO vol/vol); closed circles indicate cells stimulated in 10^{-8} mol/L fMLP. The mean percentage $\pm$ SD of cells exhibiting a specific rate for four experimental trials is shown. For control cells, $n = 363$ total cells in four trials; for 10^{-8} mol/L fMLP, $n = 376$ cells.

State of actin polymerization. We next determined the percentage of G-actin, ie, the percentage of total cellular actin that is in the globular (G-actin) form, in uninduced and DMF-induced HL-60 cells by using the DNase I inhibition assay.¹¹ Induced myeloid maturation of HL-60 cells caused no change in the percentage of G-actin (Table 1), although the total actin content significantly increased during maturation.¹⁰ The $\bar{m}$ ROL of day 5 HL-60 cells is significantly higher than the $\bar{m}$ ROL of uninduced HL-60 cells, thereby suggesting that factors other than the polymerization state of actin are important for the development of motility during myeloid maturation.

The state of polymerization of actin in HL-60 cells was then determined after stimulation with 10^{-8} mol/L fMLP by using the DNase I inhibition assay and FACS analysis of NBD-phalloidin-stained cells to estimate the relative content of F-actin.⁹ Uninduced HL-60 cells showed no significant change in the percentage of G-actin after fMLP stimulation and no change in the F-actin content as measured by fluorescence of NBD-phalloidin-stained cells. However, fMLP stimulation of induced HL-60 cells caused changes in the state of actin polymerization (Fig 3). Day 3 cells showed a 16.6% decrease in the percentage of G-actin with stimulation. By day 5, stimulation caused a 24.5% decrease in the percentage of G-actin. Stimulation of day 5 HL-60 cells with 10^{-8} mol/L fMLP caused an increase in cell fluorescence (a similar increase in fluorescence was noted for day 4 cells—data not shown), thereby indicating an increase in the relative F-actin content with fMLP stimulation (Fig 4). These data indicate that in induced HL-60 cells fMLP stimulation causes actin polymerization; however, this reorganization of the cellular cytoskeleton is not associated with a chemokinetic response.

DISCUSSION

Many of the reported biochemical changes that occur during induced myeloid differentiation of HL-60 cells temporally are related to the development of motility and chemotaxis. With increased binding of chemotactic peptides, induced HL-60 cells have shown greater responsiveness as indicated by orientation toward a chemotactic gradient⁵ and chemotaxis.⁵⁻⁷ Protein synthesis occurs during induced myeloid maturation, including changes in the pattern of expression of membrane glycoproteins,¹² an increased rate of actin synthesis, and expression of a 205,000-molecular weight protein presumed to be myosin.¹⁰ Finally, maturing HL-60 cells are more adherent to glass than are uninduced cells.⁵

Table 1. Effect of Differentiation of HL-60 Cells on the Percentage of G-Actin

Day	n	Unstimulated	10^{-8} mol/L fMLP
0	6	71.5 $\pm$ 10.3	67.9 $\pm$ 10.2
1	4	78.2 $\pm$ 6.6	72.2 $\pm$ 9.2
3	3	61.5 $\pm$ 7.3	44.9 $\pm$ 2.3
5	4	73.2 $\pm$ 10.4	47.8 $\pm$ 7.6

The percentage of G-actin is measured by DNase I inhibition in control (unstimulated) or stimulated (10^{-8} mol/L fMLP) HL-60 cells. Days 0, 1, 3, and 5 indicate the length of exposure to DMF.

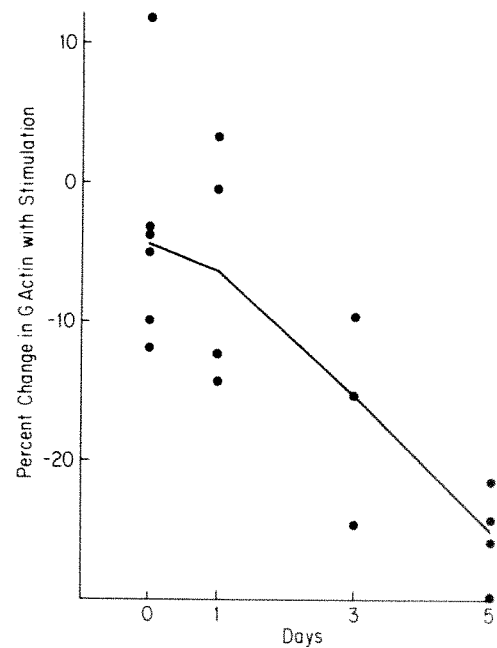


Fig 3. Change in the percentage of G-actin measured by DNase I inhibition after stimulation with 10^{-8} mol/L fMLP. Days 0 to 5 indicate the length of exposure of HL-60 cells to DMF. Each point represents the change in percentage of G-actin for a single trial. The solid line connects median values for each day.

We report that (a) the state of actin polymerization does not change with induced maturation; (b) during DMF-induced myeloid maturation, HL-60 cells become motile and have an $\bar{m}$ ROL similar to normal human neutrophils; (c) our DMF-induced HL-60 cells do not demonstrate a chemokinetic response to fMLP stimulation; and (d) stimulation of DMF-induced HL-60 cells with fMLP causes actin polymerization (similar chemotactic peptide stimulation has no effect on the state of actin polymerization in uninduced HL-60 cells).

We have previously reported that the total actin content

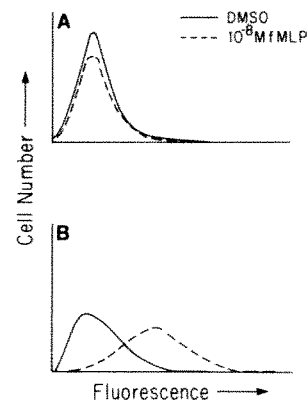


Fig 4. FACS analysis of HL-60 cells stained with NBD-phalloidin. The abscissa is the fluorescence intensity; the ordinate is the number of cells. Day 0 (A) indicates cells not exposed to DMF. Day 5 (B) indicates cells exposed to DMF for five days. Solid lines indicate cells exposed to DMSO (0.1%, vol/vol); broken lines indicate cells stimulated with 10^{-8} mol/L fMLP. Fifty thousand cells were analyzed in each condition.

increases at least twofold during induced maturation of HL-60 cells.¹⁰ In that the percentage of G-actin in nonstimulated HL-60 cells does not change during induced myeloid maturation, the basal polymerization state of actin is not the critical factor in the development of motility during myeloid maturation.

Fontana et al.,⁵ Collins et al.,⁶ and Wood and Douglas⁷ showed that HL-60 cells induced with various maturation agents became motile and that the responses to fMLP stimulation were defective. Fontana et al.⁵ demonstrated that, although the extent of induced HL-60 cell migration was similar to that of PMN, a much smaller proportion of the induced HL-60 cells participated in the chemotactic response. Using the agarose assay, Wood and Douglas⁷ found that random migration of PMN and induced HL-60 cells was similar (in agreement with our data) and that induced HL-60 cells had a much lower magnitude of directed migration. The motility assay used in our report measures chemokinesis (the stimulated increase in the rate of movement in the absence of a gradient) over a relatively short time at multiple intervals. The usual length of recording movement was ten minutes; however, cells were mixed with fMLP, injected into the chamber, and warmed for at least ten minutes to 37°C so that any adaptation response¹³ would occur before initiation of recording. We found that induced HL-60 cells do not exhibit a chemokinetic response to fMLP stimulation.

There are four possible explanations for this. Failure of induced cells to respond chemokinetically is not due to (a) the absence of cytoskeletal reorganization or (b) the presence of a small, undetected subset of responsive cells. A third possible explanation, loss of cellular function with increased cell passage, is also unlikely. The data from our model system are consistent with the hypothesis that induced HL-60 leukemic cells lack an intrinsic factor distal to actin polymerization that is required for chemokinesis but not for cytoskeletal reorganization.

It has been reported previously that chemotactic peptide stimulation induces actin polymerization in rabbit¹⁴ and rat¹⁵ neutrophils. In normal human PMN, fMLP stimulation causes both actin polymerization and a chemokinetic response, ie, an increase in the $\bar{m}ROL$.⁹ Our data show that fMLP stimulation causes an identical biochemical response in induced HL-60 cells. The $\bar{m}ROL$ of unstimulated induced HL-60 cells is very similar to PMN, and the degree of actin polymerization after fMLP stimulation is similar in PMN and induced HL-60 cells. However, this biochemical response of induced HL-60 cells is not associated with an increase in the ROL. Therefore, a lack of cytoskeletal reorganization (as defined by actin polymerization) cannot be the cause of a lack of chemokinesis. Surface contact and adherence could act as a direct stimulus for actin polymerization and cause maximal polymerization in induced HL-60 cells. Adherent cells could thus not respond to additional stimulation with fMLP by increasing the ROL. We have not measured the relative F-actin content in adherent HL-60 cells and cannot rule out this possibility. However, normal human PMN adherent to glass increase their ROL¹⁶ and the relative amount of polymerized actin (T.H. Howard, unpub-

lished observation, 1986) when exposed to fMLP. HeLa cells adherent to plastic¹⁷ also respond to stimulation with increases in polymerized actin. Since we have observed that state of actin polymerization of human PMN is not always directly related to rate of cellular locomotion (T.H. Howard, unpublished observation, 1986), it is reasonable to assume this situation exists for differentiated HL-60 cells.

The second hypothesis is that since all HL-60 cells do not show an increase in fMLP receptors with differentiation, an fMLP receptor-positive subset of the cell population accounts for the measured decrease in G-actin levels. The chemokinetic response of such a subset could be masked if the chemokinetic response were expressed as an increase in $\bar{m}ROL$ for an entire population of cells. Indeed, Nidel and coworkers⁴ have shown that less than one third of induced HL-60 cells binds a fluorescent chemotactic peptide. Our data suggest that a subset of fMLP receptor-positive cells cannot explain the defective chemokinetic response of the HL-60 cell. Using a single-cell assay analyzing over 300 individual cells, we were unable to identify a subpopulation of chemokinetically responsive HL-60 cells. Histograms of ROL before and after fMLP stimulation were virtually identical (Fig 2). Second, if a portion of induced HL-60 cells showed actin polymerization in response to fMLP stimulation, the pattern of NBD-phalloidin staining should be bimodal. One subset would show no change in the F-actin content after fMLP stimulation (few receptors), and one subset would show increases in the F-actin content (many receptors). As shown in Fig 4, the curve of fluorescence after fMLP stimulation is unimodal. These results show that the defective chemokinetic response is not due to an undetected subset of responsive cells.

The third hypothesis is that a loss of cellular function occurs with multiple cell passages. Dr James Nidel (personal communication, 1986) has noted that in late passage of HL-60 cells the induced phenotype changes and differentiated cells lose inducible chemotaxis, although they still reduce NBT. Although cells used in our experiments were passaged less than 50 times, we did not perform parallel assays for chemotaxis and chemokinesis and thus cannot rule out this possibility. However, the ROL was consistently reproducible for cells passaged over a year apart, and non-stimulated ROL in our induced HL-60 cells remained similar to normal human PMN. Whether the HL-60 cells used in these experiments are representative of the cell line regarding their potential for chemotaxis is not known, but our data demonstrate that the state of actin polymerization is not always directly related to the rate of cellular locomotion.

Our data suggest that some other factor (or factors) required for chemokinesis is (or are) lacking in induced HL-60 cells. Clearly, induced HL-60 cells are not normal myeloid cells. For example, induced HL-60 cells lack specific granules that contain lactoferrin and B₁₂-binding protein.^{5,18} PMNs from a patient with altered granulocyte function and recurrent bacterial infections were deficient in lactoferrin.¹⁹ These PMNs had normal random motility but impaired chemotaxis in the agarose assay, similar to that reported for induced HL-60 cells. In addition, exogenous lactoferrin normalized adherence and aggregation of the lactoferrin-

deficient PMNs. Lack of a cellular factor like lactoferrin or lack of cryptic fMLP receptors found in specific granules²⁰ could result in a defective chemokinetic response in induced HL-60 cells. Further studies are necessary to determine the cause of defective chemokinesis in HL-60 cells.

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The c-sis Gene Expression in Cells From a Patient With Acute Megakaryoblastic Leukemia and Down's Syndrome

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The c-sis gene expression in leukemia cells from a patient with acute megakaryoblastic leukemia and Down's syndrome was studied. The leukemia blasts were identified as megakaryoblasts by the platelet peroxidase reaction and the reactivity against antiplatelet monoclonal antibodies. Leukemia cells obtained from peripheral blood or bone marrow specimens before and after initiation of chemotherapy were analyzed for c-sis gene expression by the RNA-DNA dot blot hybridization. Although the level varied,

the mRNA of the c-sis gene was detected in all megakaryoblastic leukemia cells obtained at different clinical stages. The c-sis mRNA level in cells obtained after relapse was higher than that obtained before the initiation of therapy. The 25S c-sis mRNA was detected in a megakaryoblastic leukemia cell line established from this patient. The role of the expression of the c-sis gene in acute megakaryoblastic leukemia cells is discussed.

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THE c-sis GENE, which encodes the B chain of platelet-derived growth factor (PDGF),^{1,2} is the cellular counterpart to the transforming gene v-sis of simian sarcoma virus.³ The expression of the c-sis gene has been reported in human tumor cell lines,⁴⁻⁸ placental cytotrophoblasts,⁹ endothelial cells,^{10,11} and activated macrophages.¹² The c-sis gene expression in fresh human leukemia cells was also studied,¹³⁻¹⁵ and c-sis mRNA was found only in chronic myelogenous leukemia cells in blast transformation.¹³⁻¹⁵ However, expression of the c-sis gene in fresh megakaryocytes, the platelet progenitors and main source of PDGF, has not been demonstrated.

Acute megakaryoblastic leukemia is a rare childhood leukemia, is always accompanied by bone marrow fibrosis,^{16,17} and is often associated with Down's syndrome.¹⁸ It is assumed that the bone marrow fibrosis in this leukemia is promoted by the excretion of PDGF and platelet factor 4 from α granules of megakaryocytes.¹⁹ Thus, it is of interest to know whether acute megakaryoblastic leukemia cells express the c-sis gene and produce PDGF and whether PDGF has a role in the neoplastic proliferation of the megakaryocyte itself. We report here that the c-sis mRNA was expressed in fresh megakaryoblastic leukemia cells and a cell line established from this patient.

MATERIALS AND METHODS

Immunofluorescence assay. Leukemic cells were separated from heparinized peripheral blood by Ficoll-Hypaque density centrifugation and used for analysis including the formation E, EA (IgG-Fc), and EAC rosettes and the presence of surface immunoglo-

bins (sIg). The cell surface phenotype was analyzed with indirect immunofluorescence using monoclonal antibodies OKT3, OKT4, OKT6, OKT8, and OKT11 for T cell markers; B1 and B4 for B cell markers; J5 for common acute lymphocytic leukemia (ALL) antigen; I2 for Ia-like antigen; and MY4, MY7, and MY9 for myelomonocytoid antigens (monoclonal antibodies for T cell markers were purchased from Ortho Pharmaceutical Corp, Raritan, NJ, and the others were from Coulter Immunology, Hialeah, FL). Platelet-specific antigen was studied with the antiplatelet antibodies P2 (anti-GpIIb-IIIa, Immunotech, Marseilles, France) and KOR-P77.²⁰

Leukocyte isolation. Leukemic cells were obtained from bone marrow and peripheral blood specimens before chemotherapy and after relapse. Informed consent was obtained from the parents of the patients and adult volunteers. The cells were separated from erythrocytes by centrifugation on a Ficoll-Hypaque discontinuous density gradient at 400 g for 20 minutes at room temperature. Platelets (less than 20,000/ μ L in peripheral blood) were carefully removed by washing cells repeatedly. Normal lymphocytes were separated from peripheral blood of an adult volunteer in the same way. The cells were immediately frozen and stored in liquid nitrogen until analysis.

Preparation of RNA and hybridization. The quick blot system developed by Bresser et al²¹ was slightly modified²² and used for mRNA immobilization on nitrocellulose filter and dot blot hybridization. Preparation of poly(A)-RNA and Northern blotting analysis were done as described by Colamonici et al.²³ The PstI 1.3-kb restriction fragment of simian sarcoma proviral DNA, which contains the v-sis region² (provided by the Japanese Cancer Research Resources Bank), was used for the dot blot and Northern blot hybridizations as a probe. A 3' untranslated fragment of a mouse cytoskeletal β -actin gene²⁴ was used as a probe for the quantification of mRNA on the dotted filter. The hybridization was performed as described previously.²² Autoradiography was performed for three to seven days with an intensifying screen at -70°C.

RESULTS

Case report. A 10-month-old boy with Down's syndrome was referred to Chiba University Hospital in July 1985. Complete blood counts on admission were as follows: WBC, 1,400/ μ L with 62% lymphocytes, 13% neutrophils, and 25% blasts; hemoglobin, 7.6 g/dL; platelets, 20,000/ μ L. The bone marrow aspirates showed decreased cellularity and contained 12% blast cells, which were considered possible megakaryoblasts. Most of the blasts were 10 to 14 μ m in diameter and had a narrow basophilic cytoplasm with projections (Fig 1). The blasts were negative for myeloperoxidase and alpha-naphthyl butyrate esterase activity, but positive for alpha-naphthyl acetate esterase and acid phosphatase activity. A

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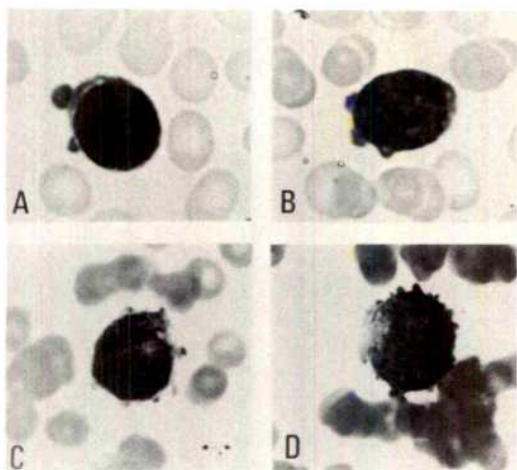


Fig 1. May-Grünwald-Giemsa preparations of blast cells from peripheral blood (A and B) and bone marrow (C and D) at time of diagnosis.

trephine biopsy sample showed both hypocellular and hypercellular areas. Atypical megakaryocytes were surrounded by increased reticulin fibers. On the basis of the platelet peroxidase²⁵ and antiplatelet monoclonal antibody examination the patient was diagnosed as having acute megakaryoblastic leukemia, and we started chemotherapy. He obtained a complete remission after 1 month but relapsed in the bone marrow in October 1985 and died in November 1985.

Cytogenetic studies. Chromosome analyses were performed on the blast cells of the bone marrow aspirate. Thirty-three metaphases were analyzed by the Giemsa banding method, of which ten cells showed 47,XY,+17p+, and nine cells showed 48,XY,+21,+17p+,+marker. The additional portion of the short arm of chromosome 17 seemed to be derived from chromosome 11. The remaining 14 cells showed 47,XY,+21, which had been observed in peripheral blood cells cultured for 72 hours with phytohemagglutinin.

Electron microscopic and immunochemical examination. Platelet peroxidase staining was done by using the

method of Breton-Gorius et al¹⁷ and was positive in the nuclear envelope and the rough endoplasmic reticulum (Fig 2). Myeloperoxidase was completely negative in the leukemic cells.

Leukemic cells formed neither E nor EA rosettes, bore no sIg, and were not reactive with anti-T cell, anti-B cell, J5, 12, and antimyelomonocytoid antibodies. However, 90% of the leukemic cells formed EAC rosettes, and 40% were strongly reactive against the antiplatelet monoclonal antibodies P2 and KOR-P77 (data not shown).

The c-sis gene expression in megakaryoblastic leukemia cells. The leukemic cells were obtained from the patient before and after the initiation of chemotherapy. Because it was very difficult to obtain enough cells from such a patient for the isolation of mRNA, we used a quick blot method for RNA-DNA dot blot hybridization.^{21,22} C-sis mRNA was detected in all megakaryoblastic cells obtained at different clinical stages, whereas normal lymphocytes and other types of leukemia cells did not express c-sis mRNA at a measurable basal level (Fig 3A). Validation of the differences in c-sis mRNA levels was provided by quantification of the β -actin mRNA on the same filter (Fig 3B). The amounts of c-sis mRNA in cells obtained from the bone marrow (45% blasts, lane 3) and peripheral blood (90% blasts, lane 4) after bone marrow relapse were at least fivefold more than those obtained before initiation of chemotherapy (90% blasts, lane 2). The cells obtained from another megakaryoblastic leukemia patient also expressed c-sis mRNA (lane 5). To obtain enough mRNA for further analysis we cultured the original blasts and established a megakaryoblastic cell line (CMK) that shared the chromosomal change, der(17)t(11;17), with the original blasts (Sato et al, submitted). Figure 3C shows that the 25S c-sis transcript was expressed in the CMK cell line.

DISCUSSION

This report is the first demonstration of the c-sis gene expression in freshly isolated megakaryoblastic leukemia cells. The c-sis expression in fresh hematopoietic cells has

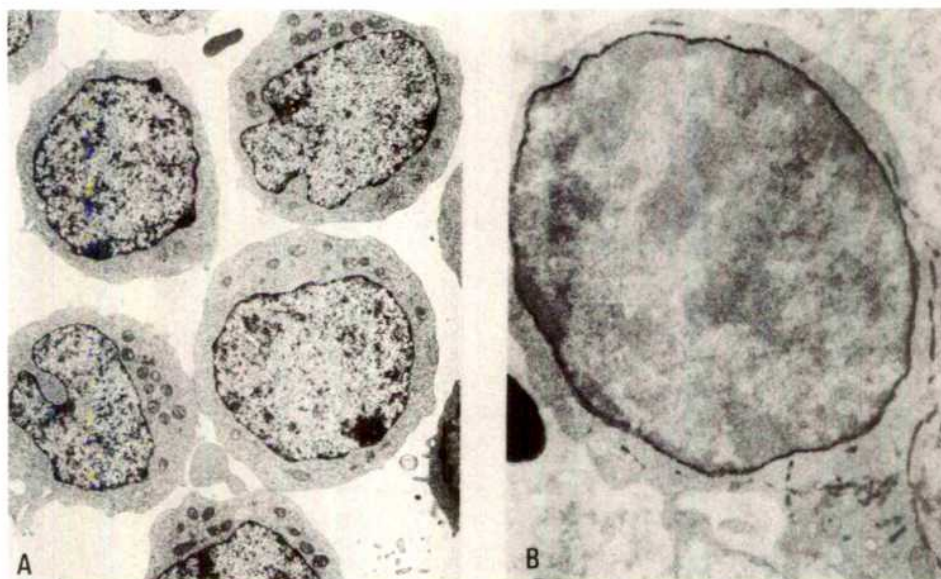


Fig 2. (A) Electron microscopic morphology of leukemic cells. Uranylacetate-lead citrate stain. (Original magnification $\times 6,400$; current magnification $\times 4,800$.) (B) Platelet peroxidase of leukemic cell, no staining. (Original magnification $\times 21,000$; current magnification $\times 15,750$.)

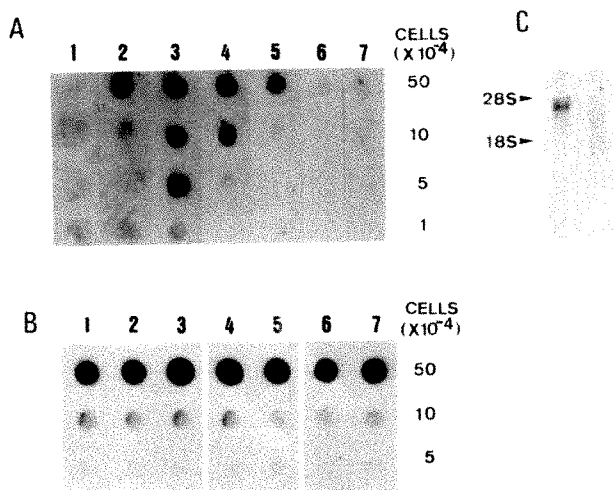


Fig 3. Detection of *c-sis* mRNA. (A) Quick blot analysis of *c-sis* mRNA. mRNA was immobilized on nitrocellulose filters, and hybridization was carried out with the *v-sis* DNA probe as described in Materials and Methods. Lane 1, normal leukocytes; lane 2, leukemic cells before initiation of chemotherapy (obtained from peripheral blood [over 90% blasts]); lane 3, leukemic cells after relapse (obtained from bone marrow [45% blasts]); lane 4, leukemic cells obtained from peripheral blood (90% blasts); lane 5, leukemic cells obtained from bone marrow (90% blasts) of another acute megakaryoblastic leukemia (5-month-old boy with Down's syndrome); lane 6, acute lymphocytic leukemia cell; lane 7, acute myeloblastic leukemia cells. (B) Quick blot analysis of cytoskeletal β -actin mRNA. The same filter was probed with mouse cytoskeletal β -actin under low stringent conditions. (C) Northern blot analysis of *c-sis* mRNA. Lane 1, a megakaryoblastic cell line, CMK; lane 2, normal peripheral lymphocytes.

been reported previously in cells from a patient with chronic myelogenous leukemia (CML) in the accelerated or blast phases.¹³⁻¹⁵ A megakaryoblastic leukemia cell line was established from bone marrow cells of a patient with CML in blast crisis,²⁶ and the *c-sis* gene was expressed when CML-derived K-562 cells were induced to differentiate to megakaryocyte. Thus, cells from CML that expressed the *c-sis* gene¹⁵ possibly differentiated along the megakaryocytic lineage.

The expression of the *c-sis* gene in megakaryoblastic leukemia cells raises the possibility that PDGF or a PDGF-like growth factor was produced in these cells. Bone marrow fibrosis is associated with a variety of hematologic malignancies including megakaryoblastic leukemia. The topographical relationship between fibrosis and megakaryocytes has been reported by Breton-Gorius et al¹⁹ who assumed that the excretion of PDGF and platelet factor 4 into the bone marrow would favor the proliferation of fibroblasts and secretion of collagen. Our results are consistent with this hypothesis. It would therefore be interesting to know whether megakaryoblastic leukemia cells secrete platelet-type PDGF.

The autocrine regulation of growth by PDGF or PDGF-like growth factors has been postulated for osteosarcoma,⁷ trophoblasts,⁹ simian sarcoma virus-transformed cells,²⁷ and rat smooth muscle cells.²⁸ The role of *c-sis* gene products in the proliferation of megakaryoblastic leukemia cells remains to be elucidated.

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Characterization of a New Human Diploid Myeloid Leukemia Cell Line (PLB-985) With Granulocytic and Monocytic Differentiating Capacity

By Kent A. Tucker, Michael B. Lilly, Louis Heck, Jr, and Thomas A. Rado

A new human diploid cell line, designated PLB-985, has been established from the peripheral blood of a patient with acute nonlymphocytic leukemia (ANLL). Cells of this line are capable of granulocytic and monocytic maturation in the presence of inducing agents. By morphology, the analysis of surface antigens, and cytochemical staining PLB-985 cells are myelomonoblasts. Transmission electron microscopy reveals them to be devoid of neutrophilic primary or secondary granules and to have an open chromatin pattern with frequent nucleoli. The modal karyotype of the line is 46,XX, with no consistent marker chromosomes or recognizable translocations. Myelomonoblasts of this line form colonies in soft agar and induce tumors (chloromas) in nude mice. Growth of the cells in the presence of dimethyl sulfoxide, *cis*-retinoic acid, or dibuty-

ryl cyclic adenosine monophosphate results in granulocytic maturation as determined by morphology, histochemical staining characteristics, and incorporation of ³⁵S-methionine into the neutrophil primary granule proteinases elastase and cathepsin G. The tumor-promoting phorbol ester phorbol myristate acetate induces PLB-985 cells to differentiate as monocytes. Cells grown in the presence of this agent rapidly become adherent to plastic, display markedly increased phagocytosis of latex particles, stain positively for α -naphthyl acetate esterase, and lose the ability to synthesize the neutrophilic proteinases. Induction of differentiation along either pathway is accompanied by a marked decrease in *myc* oncogene transcription.

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THE ACUTE HUMAN NONLYMPHOCYTIC leukemias (ANLL) are characterized by the presence, in bone marrow and peripheral blood, of myeloid cells that are morphologically immature and whose proliferation eventually replaces normal myelopoiesis. In spite of their proliferative nature in vivo, however, most fresh isolates of ANLL cells are capable of only a limited number of cell divisions in culture.¹ The development of myeloid cell lines has been difficult and at present only a few well-characterized lines exist.² The majority of these have limited differentiating capacity. The lines that have proved most valuable to leukemia research are those capable of some degree of maturation in response to differentiation-inducing agents.

The human promyelocytic cell line HL-60, for example, is capable of displaying granulocytic or monocyte/macrophage characteristics after exposure to a variety of agents.^{3,4} Similarly, the more primitive line K562 can be induced to express attributes of erythroid cells⁵ or megakaryocytes⁶ after appropriate induction. Although the study of these lines has contributed significantly to our understanding of hematopoietic cell proliferation and differentiation, their usefulness is limited by their inability to achieve a state of complete, terminal maturation indistinguishable from their normal counterparts. Thus, each myeloid line provides a model to study events surrounding particular stages of maturation.

The development of new, inducible, human myeloid cell lines with unique characteristics will permit the study of events earlier or later than has thus far been possible.

We have established a new human, diploid, myeloid leukemia cell line, designated PLB-985, obtained from the peripheral blood of a patient with refractory myeloblastic leukemia. Phenotypically these cells are myelomonoblasts. They have the capacity for both granulocytic and monocyte/macrophage maturation in the presence of inducing agents. This report documents the establishment and characterization of the new cell line.

MATERIALS AND METHODS

Case report. P.L., a 38-year-old white woman, presented in February 1985 with acute myelogenous leukemia. Her peripheral leukocyte count was $12.9 \times 10^9/L$. The differential included 13% myeloblasts, 1% promyelocytes, 1% myelocytes, 3% metamyelocytes, 2% band forms, 49% segmented neutrophils, 30% lymphocytes, and 1% monocytes. Her hemoglobin concentration was 6.7 g/dL, and her platelet count was $20 \times 10^{12}/L$. Bone marrow examination revealed myeloid hyperplasia with 43% myeloblasts, 5% promyelocytes, and decreased levels of megakaryocytes. Cytochemical studies indicated that the leukemic cell population was positive for Sudan black stain and myeloperoxidase activity. Approximately a third of the cells displayed nonspecific acid esterase activity, and no staining with PAS was detected. Surface markers were positive for OKM1 (42%) but negative for HLA-DR, natural killer cells (NK), and B1 antigens. NK antibodies used included: OKT3, Leu-19, and Leu-11. Cytogenetics revealed a normal 46,XX karyotype in all metaphases available for examination.

The patient initially was induced into a complete remission with standard chemotherapy. Subsequently she relapsed and proved refractory to all further chemotherapy. Cells obtained at that time were used to establish the present cell line.

Establishment of the myelomonoblast cell line PLB-985 in suspension culture. During the patient's final hospitalization mononuclear cells were isolated from a sample of peripheral blood by Ficoll-Hypaque density centrifugation. The cells were washed once and resuspended in Iscove's modified Dulbecco's medium (IMDM)/RPMI 1640 (7:3) media containing 10% heat-inactivated fetal calf serum (FCS). A portion of the cells was resuspended in RPMI with 10% FCS and 10% dimethyl sulfoxide (DMSO) at a concentration of 1×10^7 cells/mL for cryopreservation in liquid nitrogen vapor. Two weeks later a 1-mL aliquot of frozen cells was removed, thawed,

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washed, resuspended in IMDM/RPMI (7:3) plus 10% FCS, and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cytochemical staining. The cells were observed with Wright's, Sudan black, PAS, nonspecific acid esterase, naphthol AS-D chloroacetate esterase, and neutrophil alkaline phosphatase stain. Supravital staining was performed with acridine orange (AO) and examined with fluorescence microscopy.^{7,8}

Cell surface markers. Cultured PLB-985 cells were reacted with a panel of mouse monoclonal antibodies against B cell and myelomonocytic antigens⁹ and against the specific megakaryocytic marker PBM.¹⁰ After incubation with the monoclonal antibodies, cells were stained with affinity-purified goat antimouse IgG antibodies labeled with fluorescein isothiocyanate (Southern Biotechnology, Birmingham, AL) and examined by fluorescence microscopy or flow cytometry.

Ultrastructural studies. Preparation of PLB-985 cells for transmission electron microscopy was performed according to Roth et al.¹¹

Chromosome analysis. Bone marrow cells obtained from the patient at the time of diagnosis were cultured in RPMI 1640 media with 10% FCS for 24 hours at 34°C, after which they were exposed to colcemid (0.06 µg/mL) for 2.5 hours at 4°C. Cultured PLB-985 cells were similarly processed. Routine methods were used for culture harvest, slide preparation, and Giemsa banding of chromosomes after trypsin digestion. Direct examination of metaphase chromosome preparations was also performed.

Growth in semisolid medium. PLB-985 cells from suspension cultures were suspended in a medium of IMDM containing 20% FCS, 20 mmol/L glutamine, 1% penicillin/streptomycin, and 0.3% agar at a final cell density of 5×10^3 /mL. The plates were incubated at 37°C in a 5% CO₂ atmosphere for ten to 14 days. Colonies (>40 cells) were evaluated with an inverted-stage microscope.

Metabolic labeling of intact cells with ³⁵S-methionine. Metabolic labeling of PLB-985 cells was performed as previously described¹² with the following modifications. Preincubation and incubation were carried out in medium containing 5% Nu-Serum (Collaborative Bioresearch, Bethesda, MD) instead of FCS. Cells were lysed in a buffer consisting of 50 mmol/L Tris, pH 7.5, 1% sodium deoxycholate, 1% NP-40, 150 mmol/L NaCl, and 0.02% sodium azide. Diisopropyl fluorophosphate was added to a final concentration of 20 mmol/L immediately before use.

Immunoprecipitation of elastase and cathepsin G from cell extracts. Polyclonal and monoclonal antisera to human neutrophil cathepsin G (HNCG) and human neutrophil elastase (HNE) were obtained as previously reported¹³ by using purified HNE and HNCG isolated from normal human neutrophils.¹⁴ Antisera were stored at -80°C at 1 mg/mL in phosphate-buffered saline (PBS). Staphylococcal protein A bound to sepharose beads was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). ¹⁴C-protein molecular weight markers were from Amersham Corp (Arlington Heights, IL).

Immunoprecipitations were performed as previously described¹⁵ with some modification. For each reaction, a volume of lysate containing 5×10^7 acid-precipitable counts was diluted to 300 µL with lysis buffer. Antiserum was added at a 1:5 dilution and incubated overnight at 4°C with constant agitation. At the end of this incubation, 50 µL of protein A-Sepharose (33% wt/vol suspension in PBS) was added and incubation continued for one additional hour at 4°C. The protein A-sepharose-antibody complexes were recovered by gentle centrifugation and washed according to Dunphy et al.¹⁶ The immunoprecipitated proteins were solubilized in 100 µL treatment buffer (4% sodium dodecyl sulfate, 20% glycerol, and 125 mmol/L Tris, pH 6.8). The samples were boiled for three minutes and the Sepharose beads removed by centrifugation. The supernatants were analyzed by vertical electrophoresis through a linear 5%

to 20% SDS-polyacrylamide gradient gel prepared according to Laemmli.¹⁷ The gels were stained with Coomassie blue and treated with En³Hance (New England Nuclear, Boston). Fluorography was carried out at 80°C on Kodak XAR-5 film for 48 to 96 hours.

Induction of differentiation. Induction of granulocytic differentiation was achieved by using 1.25% DMSO (Fluka, Ronkonkoma, NY),¹⁸ 1 µmol/L *cis*-retinoic acid (Sigma Chemical Co, St Louis),¹⁹ and 750 µmol/L N⁶, 2'-dibutyl adenosine-3',5'-cyclic monophosphate (Bt₂cAMP, Sigma).²⁰

Induction of monocytic differentiation was achieved by using 50 nmol/L phorbol-12-myristate-13-acetate (PMA, Sigma).²¹

Extraction of cellular RNA and blot hybridization. Total cellular RNA was extracted from PLB-985 cells by rapid lysis in guanidinium isothiocyanate²² followed by centrifugation through 5.7 mol/L cesium chloride.²³ The purity and molecular weight of RNA fractions were routinely assessed by electrophoresis through 1.4% agarose gels containing 10 mol/L methyl mercury hydroxide,²⁴ with intact ribosomal RNA (18S and 28S) used as standards. For blot hybridization experiments formamide-denatured RNA was separated in a formaldehyde/agarose gel.²⁵ After electrophoresis the nucleic acid was transferred to nylon membrane (GeneScreen Plus, New England Nuclear) by capillary blotting.²⁶ Prehybridization and hybridization conditions were as recommended by the manufacturer. Purified DNA probes for γ -actin²⁷ and *c-myc*²⁸ were labeled with α -³²P-dCTP (deoxyeytidine triphosphate) by the random oligonucleotide method of Feinberg and Vogelstein.²⁹

RESULTS

Cell culture. The cells did not proliferate during the first 3 to 4 weeks in culture but began a gradual acceleration thereafter. RPMI 1640 medium with 10% heat-inactivated FCS was added as needed initially. By week 6 in culture, growth had stabilized, and the cells required twice-weekly passages. The new cell line, designated PLB-985, was rapidly adapted to growth in RPMI 1640 plus 5% Nu-Serum. No feeder layers or growth factor-conditioned medium was required for initiation or maintenance of the culture. The cells have been maintained in continuous suspension culture for >90 passages over 14 months. They have a doubling time of 24 to 48 hours (Fig 1) and are able to form colonies in soft agar with a plating efficiency of 0.05. Tumors (chloromas) have appeared in nude mice when these cells are implanted subcutaneously. Cells taken from PLB-985 murine tumors have been readapted to in vitro growth and display the same cytochemical and antigenic characteristics as the original line (data not shown).

Cytologic characteristics. PLB-985 cells growing in suspension culture resemble malignant myelomonoblasts. Occasional cells have blunt-ended pseudopodia. There is considerable variation in cell size, and the largest cells are often multinucleate. Typically the cells contain a large, round, or slightly irregular nucleus, fine chromatin, and one to three nucleoli (Fig 2A). The cytoplasm is scanty and deeply basophilic, and only rare azurophilic granules are present. No Auer's rods are seen. All of the cells stain positive for Sudan black but less than half for myeloperoxidase and α -naphthyl acetate esterase. Less than 10% of uninduced cells display a slight, diffuse PAS positivity. Alkaline phosphatase is not present. Supravital staining with AO reveals large, green-fluorescent, irregular nuclei with prominent

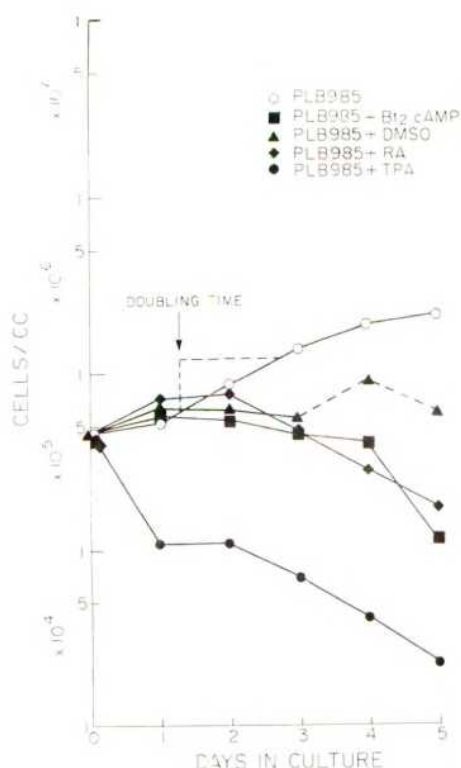


Fig 1. Growth of PLB-985 cells in the presence and absence of differentiation-inducing agents. Data points represent nonadherent cells in suspension. The concentrations of inducing agents are as described in Materials and Methods.

nucleoli and only rare cytoplasmic red-orange granular fluorescence.

Transmission electron microscopy. Ultrastructurally the PLB-985 line consists of a heterogeneous population of cells, with the predominant cell type containing a large, frequently folded nucleus, fine dispersed chromatin, and prominent nucleoli (Fig 3). The cytoplasm contains prominent rough endoplasmic reticulum and Golgi bodies, but the vast majority of the cells have no azurophilic granules.

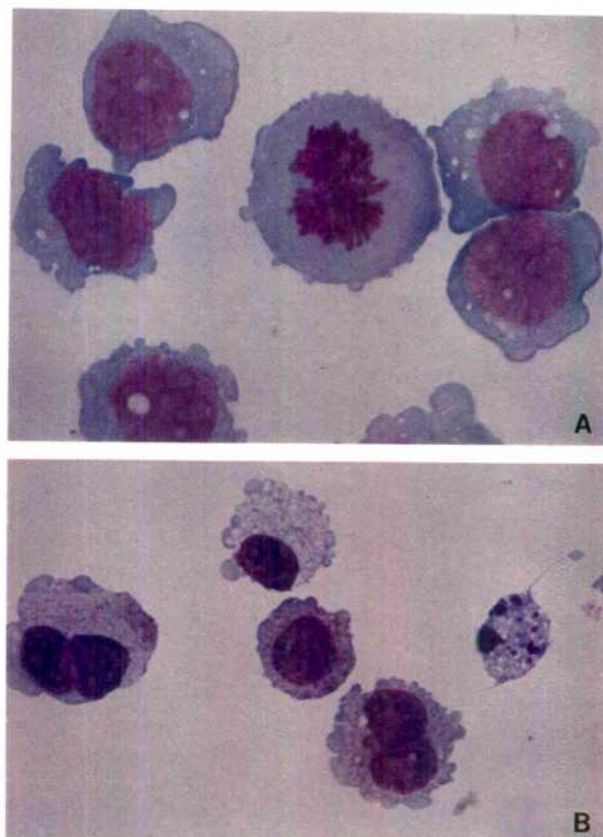


Fig 2. (A) Wright's stained preparation of uninduced PLB-985 cells in culture. Typical open nuclei, frequent nucleoli, and agranular cytoplasm are noted. A single mitotic figure is present. (Original magnification $\times 1,000$; current magnification $\times 400$.) (B) Wright's stained preparation of PLB-985 cells grown for three days in RPMI containing 5% Nu-Serum and $750 \mu\text{mol/L}$ Bt₂ cAMP. Neutrophilic maturation is indicated by increased pyknosis of nuclei, loss of nucleoli, and the appearance of azurophilic granularity in the cytoplasm. (Original magnification $\times 1,000$; current magnification, $\times 400$.)

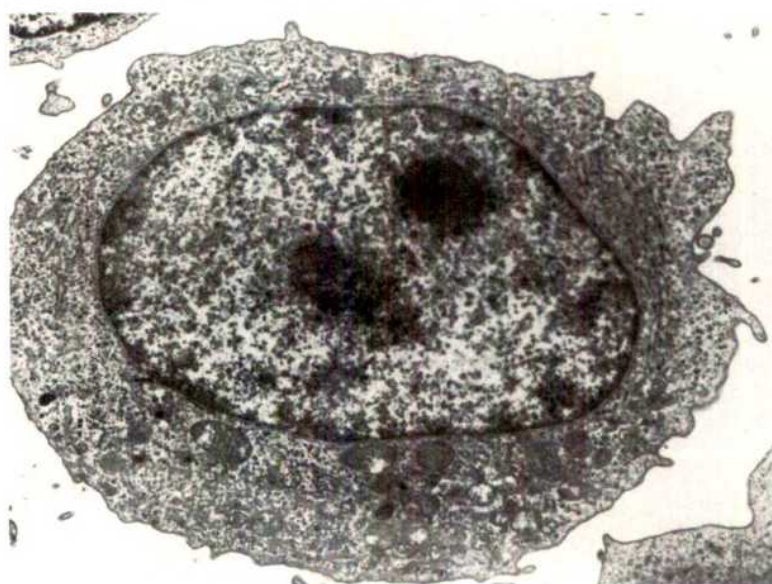


Fig 3. Transmission electron micrograph of uninduced PLB-985 cells resembles that of a myeloblast. Nucleoli are present, and there is no cytoplasmic granularity. (Original magnification $\times 19,000$; current magnification $\times 12,730$.)

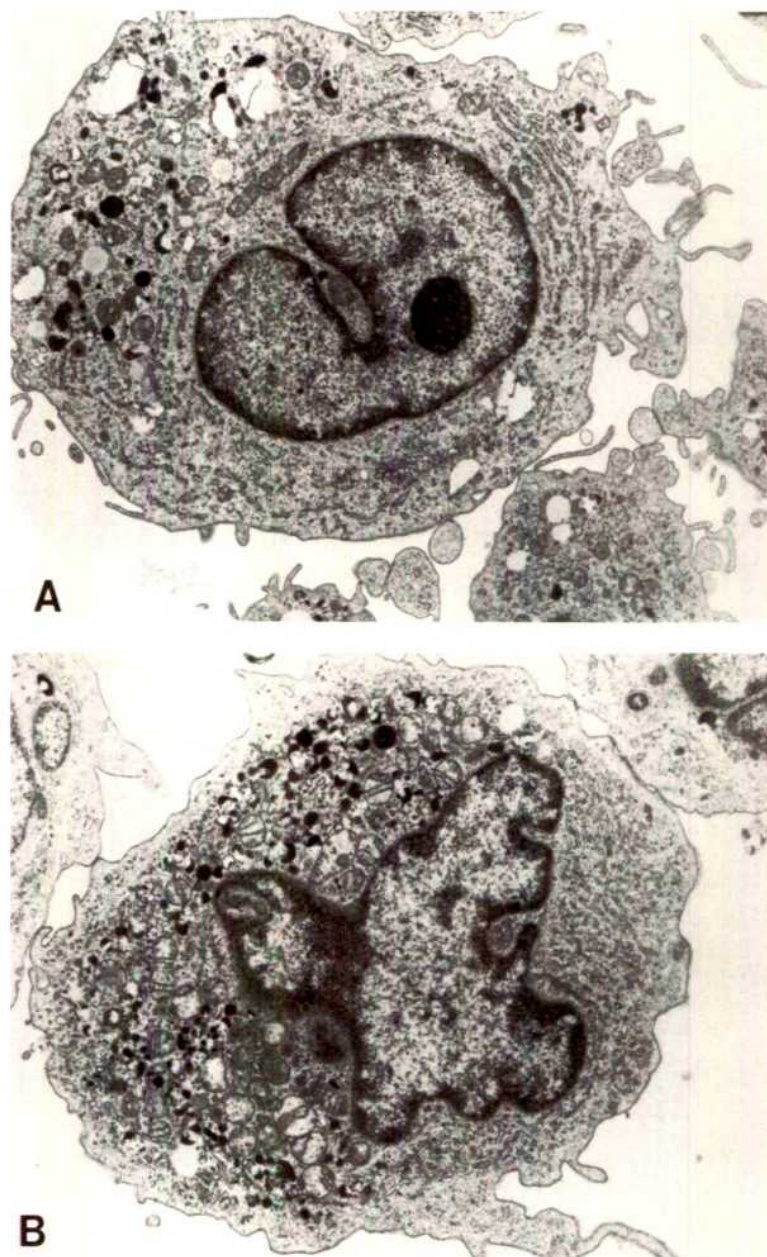


Fig 4. (A) After three days of growth in the presence of granulocytic inducing agents, cells with typical promyelocyte morphology are noted. Nucleoli are reduced in number, nuclear indentation is apparent, and the cytoplasm contains abundant electron-dense granules. (Original magnification $\times 11,000$; current magnification $\times 7,810$.) (B) Cells with the morphology of metamyelocytes appear after 72 to 96 hours in granulocytic inducing medium. Progressive nuclear maturation with disappearance of nucleoli is noted. Electron-lucent and electron-dense granules are present in the cytoplasm. (Original magnification $\times 18,000$; current magnification $\times 12,780$.)

Cell surface markers. Nearly all of the cultured cells expressed Leu-M1, and over half were positive for OKM1, MY4, MY7, and MY9 antigens. Lymphoid markers were uniformly negative, as was the specific megakaryocytic marker PBM, even in the large multinucleate cells. HLA-DR was also negative. Positive HLA antigens included HLA-A1, -A2, -B41, and -B60. The HLA phenotype matches that of the patient from whose blood the line was isolated.

Cytogenetics. Cytogenetic studies performed on cells from the patient's bone marrow at presentation revealed a normal 46,XX karyotype. The PLB-985 cells also displayed a modal karyotype of 46,XX. Many metaphases, however, have chromosomal abnormalities. None of these are specific for the cell line or present in all nuclei.

Response of PLB-985 to differentiation-inducing agents. To assess the effects of substances known to stimulate

differentiation in the human promyelocytic leukemia cell line HL-60, PLB-985 cells were cultured in the presence of various inducers and examined morphologically by Wright's stain, supravital stain with AO, and by transmission electron microscopy. In the presence of the granulocytic inducers DMSO, retinoic acid (RA), or Bt_2 -cAMP, many of the cells became spindle shaped and demonstrated granulocytic maturation by a decrease in the nuclear/cytoplasmic ratio, the appearance of nuclear indentation, and the formation of pyknotic, multilobed, and fragmented nuclei (Fig 1B). Nucleoli tend to disappear, and there is an increase in cytoplasmic azurophilic granulation.

Supravital staining of cells grown in the presence of these agents is marked by the appearance of bright red-orange cytoplasmic granular fluorescence.

Ultrastructural examination of PLB-985 after granulocytic induction revealed the presence of cells resembling

metamyelocytes and band forms (Fig 4A and 4B). Two populations of granules, and electron-dense and an electron-lucent class, are also noted in these cells. All of these changes were consistently present after treatment of the cells with any of the granulocytic inducers under the conditions described in Materials and Methods.

In the presence of the monocytic inducer PMA, PLB-985 cells became irreversibly adherent to plastic within 30 minutes to one hour of exposure. This was readily apparent ultrastructurally (Fig 5). The PMA-treated cells showed a marked increase in ability to engulf latex beads compared with either granulocytic induced or uninduced cells. These adherent cells also demonstrated strongly positive staining with α -naphthyl acetate esterase.

Growth of PLB-985 cells in suspension culture was markedly inhibited by either granulocytic or monocyte/macrophage inducers (Fig 1). After 72 hours of exposure, however, the viability of the cells remained >90% by trypan blue exclusion or supravital staining with AO.

To assess the metabolic effect of granulocytic or monocyte/macrophage maturation on the PLB-985 cell line we evaluated the biosynthesis of the primary neutrophil granule proteinases elastase³⁰ and cathepsin G.³¹ The incorporation of ³⁵S-methionine into immunoprecipitable HNE and HNCG was studied in cultured cells and in cells exposed to granulocytic inducers or PMA. For the latter experiments the small number (<5%) of nonadherent cells remaining after induction was discarded and the adherent population was labeled in situ by the addition of preincubation and incubation media directly to the culture flask.

As shown in Fig 6, PLB-985 cells growing in RPMI/Nu-Serum without inducing agents display a constitutive synthesis of elastase and cathepsin G (lanes 1 and 2). After granulocytic induction the proportion of acid-precipitable radioactivity incorporated into these proteins is increased (lanes 3 and 4). Induction of the cells with PMA results in the complete abolition of detectable elastase and cathepsin G synthesis (lanes 5 and 6). The viability of the cells under each of these culture conditions was essentially 100% as assessed by supravital staining (data not shown).



Fig 5. Monocytic maturation induced by PMA is characterized by adherence to plastic, cytoplasmic vacuolization, and the absence of granule synthesis. (Original magnification $\times 9,000$; current magnification $\times 5,580$.)

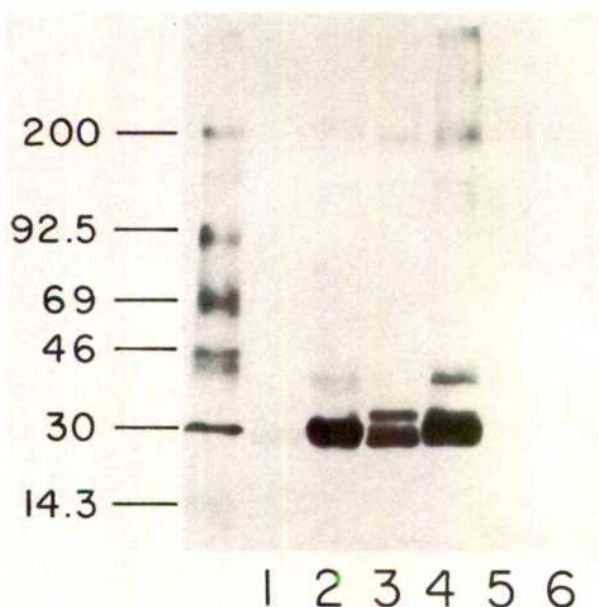


Fig 6. Fluorograph of ³⁵S-methionine-labeled PLB-985 lysates immunoprecipitated with anti-elastase or anti-cathepsin G as described in Materials and Methods. ¹⁴C-labeled proteins were used as molecular weight markers. Lanes 1 and 2, lysate of uninduced cells; lanes 3 and 4, lysate of cells induced for 72 hours with DMSO; lanes 5 and 6, lysate of adherent cells treated for 72 hours with PMA. Each lane contains 5×10^7 cpm of acid-precipitable radioactivity. In each pair the first lane was immunoprecipitated with antiserum to elastase and the second with anti-cathepsin G.

The expression of the *c-myc* oncogene has been shown to decrease after induction of differentiation in HL-60³² and in several other cell lines derived from hematologic malignancies.³³ To determine the effect of induction of *c-myc* expression in PLB-985 we extracted RNA from uninduced cells and from cells treated with PMA or Bt₂ cAMP. The same amount of RNA from each source was size fractionated, transferred to a nylon membrane, and hybridized to a radioactive *c-myc* probe as described in Materials and Methods. Differentiation in either the granulocytic or the mono-

cyte/macrophage direction resulted in a marked reduction in the proportion of the 22S *c-myc* RNA (Fig 7). Duplicate blots hybridized to purified γ -actin probe displayed no decrease in signal after granulocytic maturation and only a small decrease in RNA from cells treated with phorbol ester.

DISCUSSION

The establishment of human myeloid leukemia cell lines has provided much information about ANLL cell proliferation and differentiation. Several existing lines, which grow in culture as malignant early myeloid cells, are capable of further differentiation under the proper influences. In particular, PL-21 can undergo granulocytic maturation,³⁴ whereas KG-1, ML-1.3, and THP-1 respond to tumor promoters and other agents by maturation along the monocyte-macrophage pathway.³⁵ The HL-60 promyelocytic leukemia cell line has stimulated intense study because of its capacity to respond to different agents by manifesting either granulocytic or monocytic characteristics. The myelomonoblastic cell line described herein is also bipotential. The inducing agent to which the cells are exposed determines whether terminal differentiation proceeds along the granulocyte or monocyte/macrophage pathway.

Phenotypically and by surface marker analysis PLB-985 cells are malignant myelomonoblasts. Growing in liquid culture with a serum substitute, their capacity for self-renewal appears to be unlimited. The morphology of the cells under these conditions, with conventional stains and by electron microscopy, suggests that they are less mature than the promyelocytes of the HL-60 line. PLB-985 may thus provide the opportunity to study events involved in granulocyte/monocyte-macrophage commitment earlier than that afforded by HL-60.

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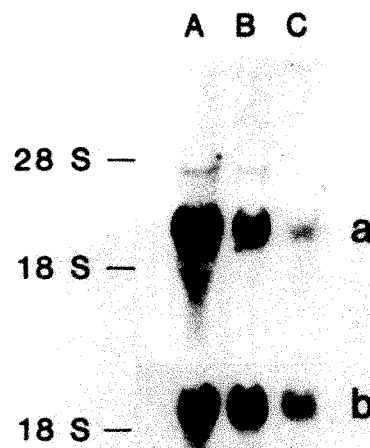


Fig 7. Hybridization of PLB-985 RNA to *c-myc* and γ -actin probe. Each lane contains 30 μ g of total cellular RNA. Sources of RNA: lane A, uninduced cells; lane B, cells induced with Bt₂-cAMP for 96 hours; lane C, adherent cells induced with PMA for 48 hours. 18S and 28S molecular weight markers are indicated. Blot a, hybridization to *c-myc*; blot b, hybridization to γ -actin probe.

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Eosinophils Do Respond to fMLP

By Maria Yazdanbakhsh, Carel M. Eckmann, Leo Koenderman, Arthur J. Verhoeven, and Dirk Roos

Eosinophils were isolated from normal human blood by separation over Percoll gradients, which resulted in eosinophil suspensions of a purity higher than 95% and recoveries of about 65%. Normal human eosinophils were found to respond to formyl-methionyl-leucyl-phenylalanine (fMLP) at concentrations $>10^{-7}$ mol/L with an increase in the concentration of intracellular free calcium, oxygen consumption, nitroblue tetrazolium reduction, and chemiluminescence. The maximal response of eosinophils to fMLP was lower than that of neutrophils isolated from the same blood samples and required at least ten times as much

fMLP as was needed for neutrophils. Low fMLP concentrations ($\sim 10^{-8}$ mol/L), which in themselves did not stimulate O_2 consumption by either eosinophils or neutrophils, primed these cells to respond to a suboptimal concentration of another stimulus. Purification of eosinophils after treatment of whole blood with fMLP showed that these eosinophils had lost their ability to respond to fMLP. We conclude that normal eosinophils do respond to fMLP and that therefore fMLP should not be used to isolate eosinophils.

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DIFFICULTIES in obtaining eosinophils from normal human blood have hampered studies with these cells. Most studies so far have used eosinophils from patients with eosinophilia; these eosinophils are different from those of normal individuals.^{1,2} In a recent report,³ a method for the isolation of normal human eosinophils based on the differential responsiveness of eosinophils and neutrophils to formyl-methionyl-leucyl-phenylalanine (fMLP) was described. According to this report, normal human eosinophils do not respond to fMLP in the concentration range of 10^{-7} to 10^{-6} mol/L. However, previous studies on eosinophils from patients with eosinophilia have shown that fMLP enhances the expression of Fc receptors and stimulates oxygen metabolite release as well as leukotriene C₄ (LTC₄) generation by these cells.^{4,6} In addition, in eosinophils from normal individuals, the generation of LTC₄ in response to opsonized particles was shown to be augmented by pretreatment of the cells with low concentrations of fMLP.⁷ In the present report, we show that normal human eosinophils are also able to respond to fMLP by an increase in intracellular free calcium, oxygen consumption, nitroblue tetrazolium (NBT) reduction, and chemiluminescence. In addition, we demonstrate that eosinophils isolated with fMLP by the method of Roberts and Gallin³ have altered properties.

MATERIALS AND METHODS

Superoxide dismutase from bovine erythrocytes, lucigenin (10,10'-dimethyl-9,9'-biacridinium dinitrate), and NBT were obtained from Sigma Chemical Co (St Louis). Fura-2 was bought from Molecular Probes (Junction City, OR). Digitonin was purchased from Merck (Darmstadt, FRG). Phorbol myristate acetate (PMA; Consolidated Midland Corp, Katonah, NY), fMLP (Vega Biochemicals, Tucson), and cytochalasin B (ICI Research Laboratories, Alderley Park, Cheshire, UK) were dissolved in dimethyl sulfoxide (DMSO) at 1,000 times the final concentration for cell incubations and stored at -70°C . The final concentration of DMSO ($10\text{ }\mu\text{g/mL}$) had no effect on the cells.

Percoll. Percoll suspensions (Pharmacia Fine Chemicals, Uppsala, Sweden) were prepared with specific gravities of 1.077, 1.082, and 1.084 g/mL at room temperature. The specific gravities were measured with a density meter (Anton Paar, Graz, Austria, model DMA 46). The osmotic value of all Percoll suspensions was 290 mosm (measured with a semi-Osmometer, Knauer, Berlin, FRG).

The incubation medium for the cells consisted of 138 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4 , 0.6 mmol/L CaCl_2 , 1.0 mmol/L MgCl_2 , 5.5 mmol/L glucose, and 0.5% (wt/vol) human albumin.

Cell preparation. Blood was obtained from healthy volunteers with 3% to 8% eosinophils in their peripheral blood. Granulocytes were purified from the buffy coat of 500 mL of blood anticoagulated with 0.4% (wt/vol) trisodium citrate (pH 7.4). The buffy coat was diluted three times with phosphate-buffered saline (PBS) containing 13 mmol/L trisodium citrate (PBS-citrate) and centrifuged (20 minutes, 1,000 g, room temperature) over Percoll with a specific gravity of 1.077 g/mL. The erythrocytes in the pellet fraction were lysed with isotonic ammonium chloride at 4°C as described.⁸ The granulocytes were subsequently incubated for 30 minutes at 37°C in Hanks' buffered salt solution with 20 mmol/L HEPES and 5% (vol/vol) fetal calf serum (pH 7.0 at 37°C), to restore the original specific gravity of the cells. Thereafter, the granulocytes were suspended in Percoll with a specific gravity at room temperature of 1.084 g/mL. This suspension was layered on top of Percoll with a specific gravity of 1.1 g/mL and overlaid with 0.2 mL PBS. The tubes were centrifuged for 20 minutes at 1,000 g at room temperature. Neutrophils ($>98\%$ pure) from the upper interface were collected, washed in PBS-citrate, and resuspended in incubation medium. The cells from the lower interface were also washed and resuspended in the incubation medium; the resulting cell suspension represented a 50% to 70% recovery of the eosinophils present in the buffy coat, at more than 95% purity. The contaminating cells were neutrophils.

Eosinophils were also isolated as described by Roberts and Gallin.³ Briefly, whole blood was anticoagulated with EDTA (final concentration, 0.3%, wt/vol). Prewarmed blood was incubated with fMLP (10^{-6} mol/L) for 15 minutes at 37°C . Thereafter, it was layered over Percoll with a specific gravity of 1.082 g/mL and centrifuged (25 minutes, 400 g, room temperature). Erythrocytes and eosinophils constituted the layer below the Percoll. The erythro-

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cytes were lysed with isotonic ammonium chloride as described in the previous paragraph. The purity of eosinophils was higher than 90%, with recoveries of 70% to 90%.

Metabolic reactions. Oxygen consumption was measured with an oxygen electrode as described before.⁹ NBT reduction was measured by incubating cells for 30 minutes at 37°C with NBT (500 µg/mL) in the presence or absence of stimuli. At the end of the incubation, the cells were fixed with paraformaldehyde (final concentration, 1%, vol/vol) and spun on a glass slide with a cytospin centrifuge. Thereafter, the slides were stained with nuclear fast red (0.1%, wt/vol), and the percentage of NBT-positive cells was scored as described before.¹⁰ One cell sample was stained with May-Grünwald/Giemsa and used for the determination of the cell composition.

Lucigenin-enhanced chemiluminescence was determined as follows: Minivials were filled with 1 mL of cell suspensions (10^5 cells/mL). The vials were placed in polyethylene counting vials in a scintillation spectrometer with a controlled temperature of 37°C (Packard Tricarb 3310, Downers Grove, IL). The reaction was started by the addition of 1 mL of prewarmed incubation medium containing lucigenin (640 µmol/L) and a stimulus. Each vial was counted continuously for ten minutes in the out-of-coincidence mode of the apparatus. Where indicated, cells were preincubated with cytochalasin B (10 µg/mL) for three minutes at 37°C.

Measurement of cytosolic free Ca^{2+} in human eosinophils and neutrophils. Eosinophils and neutrophils were washed and suspended in HEPES medium (132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 20 mmol/L HEPES, 1.2 mmol/L potassium phosphate, 5.5 mmol/L glucose, and 0.5% (wt/vol) human albumin (pH 7.4)). Cells were loaded with Fura-2¹¹ by incubation of the cells at 37°C with the acetoxy methyl ester of Fura-2 (Fura-2/AM). Prewarmed cells (10^7 cells/mL) were incubated with 1 µmol/L Fura-2/AM (from a 1-mmol/L stock in DMSO) for 20 minutes at 37°C and then diluted five times with the warm HEPES medium. The incubation was continued for a further 40 minutes. Control cells were incubated with 0.1% (vol/vol) DMSO. After the loading procedure, the cells were washed, resuspended in HEPES medium, and kept on ice before use. Prior to each measurement, Fura-2-loaded eosinophils or neutrophils (10^7 cells/mL) were diluted in HEPES medium (final concentration, 10^6 cells/mL) and incubated at 37°C for 15 minutes before transfer to a cuvette.

Fluorescence measurements were performed with a spectrofluorometer (model RF-540, Shimadzu Corp, Kyoto, Japan). The contents of the cuvette were magnetically stirred, and the cuvette holder was thermostated at 37°C. The excitation and emission wavelengths were 335 nm and 505 nm, respectively.¹¹ Calibration of Fura-2 fluorescence as a function of $[Ca^{2+}]_i$ was determined essentially as described for Quin-2 fluorescence^{12,13} with the following modifications. To saturate all trapped Fura-2 with Ca^{2+} , 5 µmol/L digitonin was added to the cell suspension. At this digitonin concentration, the cell membrane was found to be permeable for small solutes without causing lysis of the cells, thereby avoiding a dramatic shift in autofluorescence. Subsequently, the Fura-2 signal was quenched by adding 0.5 mmol/L Mn^{2+} (from a stock of 0.5 mmol/L $MnCl_2$) to the cell suspension. Both the Ca^{2+} -dependent and the Ca^{2+} -independent fluorescence signals were quenched by addition of Mn^{2+} . The percentage of Ca^{2+} -independent Fura-2 fluorescence was found to be 36% of the total Fura-2 fluorescence quenched by Mn^{2+} . Cytosolic free Ca^{2+} was calculated as follows:

$$[Ca^{2+}]_i = 224 \text{ nmol/L} \frac{F - (F_{\min} + 0.36 [F_{\max} - F_{\min}])}{F_{\max} - F}$$

where 224 nmol/L is the K_d for the Fura-2/ Ca^{2+} complex,¹¹ $F_{\max}$ is

the fluorescence after digitonin addition, and $F_{\min}$ is the fluorescence after Mn^{2+} quenching. Agonists used gave no autofluorescence changes in unloaded control cells.

Fura-2 contents in the cells were determined by adding Fura-2-free acid to the cell suspensions. On average, the Fura-2 content of the cells was 110 µmol/L, under the assumption that the free water content of eosinophils is approximately the same as that found in neutrophils, ie, 0.35 µL/ 10^6 cells.¹⁴

Statistical analysis. Statistical analysis was performed with Student's *t* test or Student's *t* test for paired observations.

RESULTS

Oxygen consumption. Normal human eosinophils responded to fMLP by a rapid increase in oxygen consumption. The response was dose dependent, reaching a maximum at about 10^{-5} mol/L fMLP. The optimal fMLP concentration for the neutrophil reaction was about 10^{-6} mol/L, and the magnitude of the maximal response was twice that shown by eosinophils (Fig 1). It has been shown previously that cytochalasin B enhances the response of neutrophils to fMLP.¹⁵ We found that the O_2 consumption by eosinophils in response to fMLP was also augmented by pretreatment of these cells with cytochalasin B. This agent by itself had no effect on the O_2 consumption of either cell type. Cytochalasin B augmented the fMLP-stimulated neutrophil respiration by 100% to 200%, whereas that of eosinophils was enhanced by 30% to 60%.

fMLP at a concentration of 10^{-8} mol/L did not stimulate O_2 consumption by eosinophils; however, it was capable of priming these cells (Table 1). Eosinophils pretreated with fMLP (10^{-8} mol/L) for two minutes showed an O_2 consumption in response to a suboptimal concentration of PMA (0.5

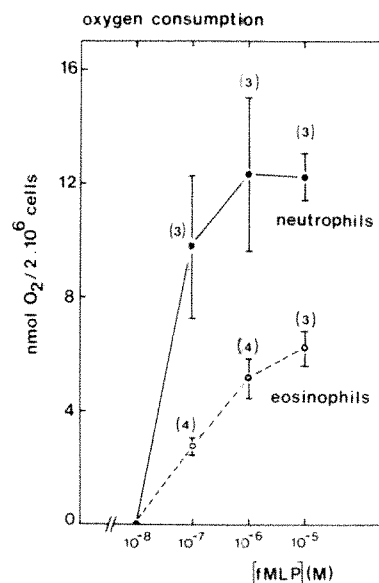


Fig 1. Oxygen consumption by eosinophils (---) and neutrophils (—) in response to various fMLP concentrations. The results show the maximum amount of oxygen consumed by cells stimulated with fMLP (usually three to four minutes after stimulation). The amount of oxygen consumed by cells at rest has already been subtracted. Each point represents the mean $\pm$ SEM of (n) experiments.

Table 1. Priming Effect of fMLP

Test Condition	Oxygen Consumption (nmol O ₂ /10 ⁶ cells/min)		Lag time (Min)	
	Eosinophils	Neutrophils	Eosinophils	Neutrophils
PBS pretreatment + PMA	1.6 ± 0.3	0.6 ± 0.2	8.4 ± 2.0	6.5 ± 0.3
fMLP (10 ⁻⁸ mol/L)				
pretreatment + PMA	8.6 ± 1.4	3.0 ± 0.4	5.4 ± 1.3	3.3 ± 0.3

Oxygen consumption by eosinophils and neutrophils in response to PMA (0.5 ng/mL for eosinophils and 1.0 ng/mL for neutrophils) is shown. The cells were preincubated with either PBS or fMLP (10⁻⁸ mol/L) for two minutes before the addition of PMA. fMLP by itself did not stimulate either cell type. The lag time is the time after the addition of PMA, before maximum velocity is reached, as defined by Cohen and Chovaniec.¹⁶ All results are expressed as means ± SEM of three experiments. The differences between PBS- and fMLP- (10⁻⁸ mol/L)-treated cells were all significant at $P < .05$ using Student's *t* test for paired observations.

ng/mL) with a shorter lag time as well as a higher maximal velocity than control cells pretreated with PBS. Similar results were obtained with neutrophils. Higher concentrations of PMA are needed for the stimulation of neutrophils as compared with eosinophils: hence the suboptimal concentration used in our experiments with neutrophils was 1 ng/mL. DMSO (the solvent of fMLP) had no priming effect.

NBT reduction. When stimulated with PMA (0.1 µg/mL), more than 95% of the eosinophils and neutrophils reduced NBT. The response of normal human eosinophils and neutrophils to increasing concentrations of fMLP is shown in Fig 2. Pretreatment of the cells with cytochalasin B (10 µg/mL) before stimulation with 10⁻⁷ mol/L fMLP increased the percentage of eosinophils capable of reducing NBT from 1% to 19% and that of neutrophils from 21% to 82%. Cytochalasin B alone did not stimulate the reduction of NBT by either cell type. When the eosinophils were primed with fMLP (10⁻⁷ mol/L) for three minutes, the percentage of formazan-positive eosinophils in response to PMA (25 ng/mL) increased from 50% to 90%.

Lucigenin-enhanced chemiluminescence. Neutrophils responded to fMLP by a rapid increase in chemiluminescence that peaked at about one to two minutes and reached a value of $8.9 \pm 1.4 \times 10^3$ cpm (mean ± SEM of nine

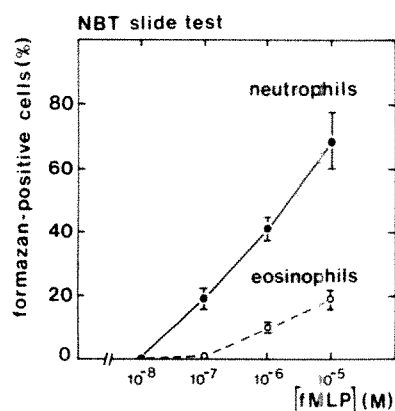


Fig 2. NBT reduction by eosinophils (---) and neutrophils (—) in response to various concentrations of fMLP. Each point represents the mean ± SEM of three experiments

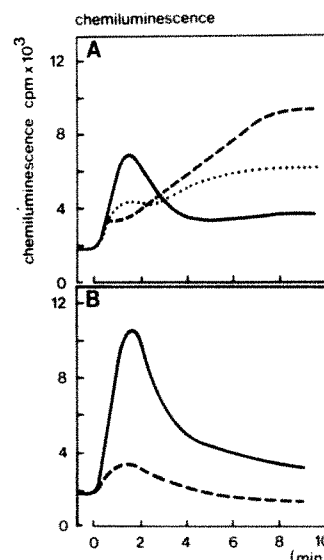


Fig 3. Lucigenin-enhanced chemiluminescence. (A) fMLP (2 µmol/L) was added at time 0 to eosinophils (---), neutrophils (—) and a 1:1 mixture of eosinophils and neutrophils (· · ·). (B) fMLP (2 µmol/L) was added to the cells preincubated with cytochalasin B (10 µg/mL). The purity of the eosinophils and neutrophils was 95% and 96%, respectively.

experiments). The eosinophil response to fMLP was biphasic; an initial small peak was followed by a slowly increasing second peak that reached a maximum of $14.1 \pm 2.3 \times 10^3$ cpm (mean ± SEM of nine experiments) at around ten minutes. Figure 3A shows that the first peak of eosinophil chemiluminescence increased and the second one decreased with increasing neutrophil contamination.

The eosinophil response to fMLP in this assay, unlike the O₂ consumption and NBT reduction, was inhibited by cytochalasin B (Fig 3B). In contrast, the neutrophil chemiluminescence with fMLP was enhanced by cytochalasin B. Chemiluminescence is an oxygen-dependent process but does not necessarily correlate with other parameters of the oxidase activity, evident from the difference in the kinetics of chemiluminescence and O₂ consumption. Thus, cytochalasin B could affect a reaction distinct from the oxygen metabolism itself. Cytochalasin B alone had no effect on the chemiluminescence of either eosinophils or neutrophils.

Cytosolic free Ca²⁺. Intracellular free calcium concentrations in both eosinophils and neutrophils were measured with the fluorescent indicator Fura-2. The change in Fura-2 fluorescence in neutrophils after the addition of fMLP (Fig 4) was similar to the change in Quin-2 fluorescence in neutrophils as observed by Pozzan et al.¹³ The resting free Ca²⁺ levels in eosinophils and neutrophils was 75 ± 6 nmol/L (n = 9) and 93 ± 6 nmol/L (n = 7), respectively (mean ± SEM of n experiments). Upon addition of 10⁻⁸ mol/L fMLP, the intracellular Ca²⁺ concentration in neutrophils rose to values of more than 1 µmol/L. In eosinophils, 10⁻⁶ mol/L fMLP also induced a very rapid increase in the intracellular Ca²⁺ concentration, to a value of 297 ± 23 nmol/L (mean ± SEM, n = 9). This response was transient (Fig 4). When eosinophils were stimulated with 10⁻⁸ mol/L

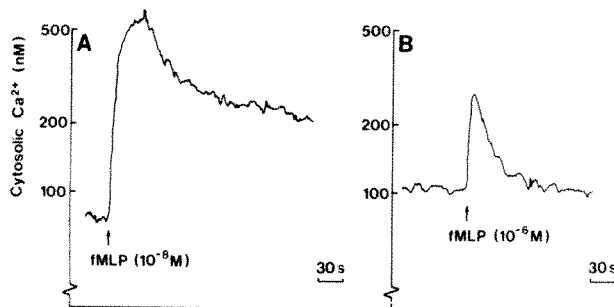


Fig 4. A representative experiment from a series of nine showing the effect of fMLP (10^{-6} mol/L) on intracellular free Ca^{2+} concentrations of (A) neutrophils and (B) eosinophils (for Ca^{2+} values, see the text).

fMLP, these cells showed no increase in the level of free intracellular Ca^{2+} .

Comparison between eosinophils isolated by two different procedures. Normal eosinophils isolated by our method were compared with the eosinophils from the same donors purified after treatment of whole blood with fMLP as described by Roberts and Gallin.³ The change in intracellular free calcium was measured as a sensitive parameter for the response to a number of stimuli. The resting intracellular free Ca^{2+} levels were 79 ± 6 nmol/L in normal eosinophils and 113 ± 12 nmol/L in the eosinophils purified after the addition of fMLP to whole blood (mean $\pm$ SEM of five paired experiments, difference not significant). After the addition of fMLP, the intracellular Ca^{2+} concentration in normal eosinophils increased significantly by 205 ± 48 nmol/L above resting levels (mean $\pm$ SEM of five experiments). In contrast, eosinophils isolated after fMLP treatment of blood had lost their ability to respond to fMLP (10^{-6} mol/L): the increase in the intracellular free Ca^{2+} was only 27 ± 13 nmol/L above resting values (mean $\pm$ SEM of five experiments). An example is shown in Fig 5A. There was no significant difference between the eosinophils isolated by the

two methods when the response to ionomycin or platelet-activating factor (PAF) was assessed (Fig 5B, C).

DISCUSSION

In this report we have shown that normal human eosinophils respond to the chemotactic peptide fMLP by an increase in free intracellular calcium, oxygen consumption, NBT reduction, and chemiluminescence. Recently, we have shown that these cells also release granule enzymes after treatment with fMLP (2×10^{-6} mol/L).¹⁷ Cytochalasin B augmented the response of both eosinophils and neutrophils to fMLP in the O_2 consumption of NBT reduction tests. Despite the capacity of normal eosinophils to exhibit an oxidative burst with PMA that is twice as high as that of neutrophils,¹⁷ their maximal response to fMLP was lower than that of neutrophils. In addition, higher concentrations of fMLP were required for an optimal eosinophil response compared with that for neutrophils. These results could suggest the presence of lower numbers of fMLP receptors on the eosinophil surface. In the NBT reduction test with fMLP (10^{-5} mol/L), 20% of the eosinophils were found to be formazan-positive. However, from the priming experiments we conclude that the large majority of the eosinophils can respond to fMLP.

The evidence that the responses seen were not due to contaminating neutrophils is as follows: (a) Eosinophil preparations with a purity $\geq 98\%$ exhibited an O_2 consumption to fMLP that was 50% of the response seen by neutrophils (one of the experiments shown in Fig 1). (b) Eosinophils did not respond to 10^{-8} mol/L fMLP with an increase in free intracellular Ca^{2+} , whereas neutrophils responded already maximally to 10^{-8} mol/L fMLP (ie, no further increase in intracellular free Ca^{2+} concentrations was seen upon stimulation with 10^{-6} mol/L fMLP). Thus, the lack of response of the eosinophil suspensions to 10^{-8} mol/L fMLP proves that the contamination with neutrophils was negligible. (c) Cytochalasin B inhibited the eosinophil chemiluminescence in

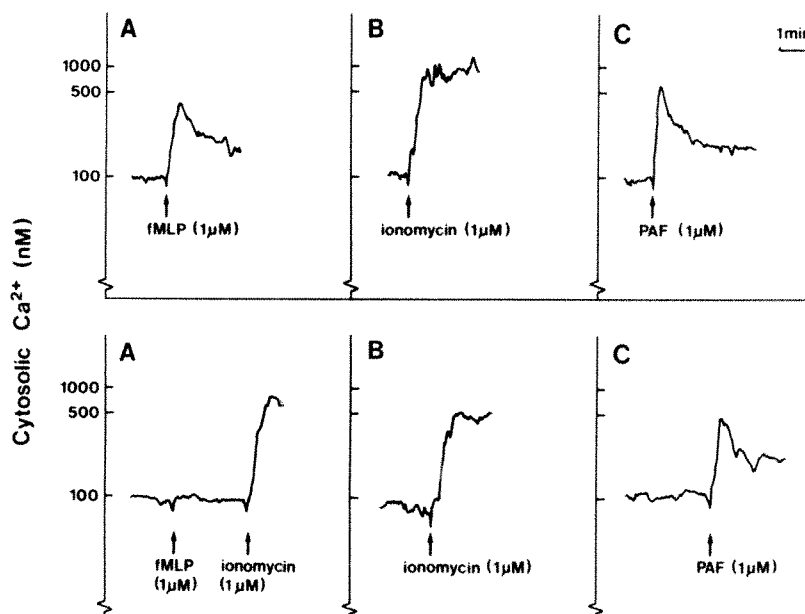


Fig 5. Intracellular free Ca^{2+} concentrations in eosinophils isolated by our method (top panel) and by the method described by Roberts and Gallin³ (bottom panel) (see Materials and Methods). Effects are shown of the addition of (A) fMLP (10^{-6} mol/L), representative of five experiments (for Ca^{2+} values, see the text); (B) ionomycin (10^{-6} mol/L), representative of five experiments (in normal eosinophils, the intracellular free Ca^{2+} level increased to 590 ± 40 nmol/L as compared with 670 ± 160 nmol/L in eosinophils purified by the method described by Roberts and Gallin³; mean $\pm$ SEM of five experiments); and (C) PAF (10^{-6} mol/L), representative of two experiments (the free intracellular Ca^{2+} increased to 354 nmol/L and 352 nmol/L in normal eosinophils and eosinophils isolated with fMLP,³ respectively; mean of two experiments).

response to fMLP while augmenting that of neutrophils. Thus, the cytochalasin B-inhibitable response is due to eosinophils (Fig 3). (d) The kinetics of the fMLP-induced chemiluminescence response of eosinophils is different from the kinetics of the neutrophil response. (e) In the NBT reduction test where morphologically identifiable cells are examined, eosinophils were seen to be able to reduce NBT in response to fMLP. In addition, work carried out in another laboratory has shown that eosinophils present in a granulocyte preparation and visualized in a chemotactic gradient were able to respond to fMLP (L. Håkansson and P. Venge, personal communication, March 1986). The possibility that products secreted by contaminating neutrophils stimulated with fMLP result in the activation of eosinophils cannot be completely ruled out. However, the observation that fMLP induces an increase in intracellular free Ca^{2+} in eosinophils without an appreciable lag time as well as our findings that very pure (>98%) preparations of eosinophils can still respond to fMLP render this possibility highly unlikely.

Recently, Roberts and Gallin³ reported a purification method for human eosinophils in which fMLP (10^{-6} mol/L) was added to whole blood. This treatment induced a decrease in the specific gravity of the neutrophils, thus facilitating the separation of eosinophils from neutrophils. The authors presume that eosinophils do not respond to fMLP and that, therefore, after treatment of blood with fMLP, the eosinophils can still be called normal. Our results contradict the

latter opinion and show that normal human eosinophils can respond to fMLP at concentrations $\geq 10^{-7}$ mol/L. An important point is that even at very low concentrations of fMLP (10^{-8} mol/L), which in themselves do not stimulate the eosinophils, these cells are primed to respond to a subsequent suboptimal dose of another stimulus (Shaw et al⁷ and this study).

In experiments where eosinophils from the same donor were isolated by either our method or by the method of Roberts and Gallin,³ we obtained evidence that human eosinophils respond to fMLP added to whole blood. This was deduced from the observation that, unlike normal eosinophils, the eosinophils obtained from fMLP-treated blood had lost the ability to respond to fMLP, presumably due to receptor desensitization.¹⁸ This altered response of the latter eosinophils cannot be accounted for by our inability to purify these cells successfully because their response to ionomycin and PAF was normal. In addition, we have isolated eosinophils by the method of Roberts and Gallin³ from random, nonselected donors with similar results to those obtained with eosinophils purified by the same method from our selected donors (with 3% to 8% eosinophils in their peripheral blood).

In conclusion, normal human eosinophils do respond to fMLP, and therefore any isolation method that involves the use of fMLP will yield altered eosinophils.

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Productive Infection by B19 Parvovirus of Human Erythroid Bone Marrow Cells In Vitro

By Keiya Ozawa, Gary Kurtzman, and Neal Young

B19 parvovirus, the cause of fifth disease and transient aplastic crisis, has been successfully propagated in suspension cultures of human erythroid bone marrow cells obtained from patients with sickle cell disease and stimulated by erythropoietin. B19 inoculation in vitro resulted in a marked decline in identifiable erythroid cells over seven to nine days of incubation. Characteristic giant early erythroid cells were seen on Wright's-Giemsa stain of infected cultures. By in situ hybridization, 30% to 40% of erythroblasts were infected at 48 hours; a similar proportion of cells showed B19 capsid protein by immunofluorescence. B19 DNA was present in erythroblasts but not in the leukocyte fraction of bone marrow. B19 replication, as determined by Southern analysis, and B19 encapsidation, as determined by sensitivity of isolated cell fractions to

DNase I, were restricted to the nuclei. B19 DNA was detectable in the nuclei from infected cultures beginning at 18 hours and in the supernatant at 32 hours; B19 genome copy number was estimated at about 25,000 to 30,000/infected cell at 48 hours. Recovery of virus depended on the multiplicity of infection (moi); at low moi, approximately 200 $\times$ input virus was recovered from total cultures and 50 $\times$ from the culture supernatants. Virus released into the supernatant was as infectious or more infectious than virus obtained from sera of infected patients. Human erythroid bone marrow culture represents a safe in vitro system for the elucidation of the cellular and molecular biology of the pathogenic B19 parvovirus.

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METHODS

Cell culture, culture conditions, and inoculation procedure. The source of B19 virus for all experiments was a sample of serum obtained from a patient with sickle cell disease and transient aplastic crisis during a recent B19 epidemic (Minor II, 60 μ g B19 DNA/mL, approximately 2×10^{13} particles/mL).⁸ Bone marrow was obtained from patients with sickle cell disease and thalassemia or from normal volunteers after informed consent according to a protocol approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute. Bone marrow mononuclear cells were infected by addition of serum at varying concentrations, diluted in Iscove's modification of Dulbecco's medium (IMDM; Flow Laboratories, McLean, VA) at a concentration of 5×10^7 cells/mL (total volume 0.4 mL). Virus was allowed to adsorb to cells for two hours at 4°C and was then diluted to 2×10^6 cells/mL in IMDM containing 20% fetal calf serum (FCS) and 1 U/mL erythropoietin (epo; step III, Connaught Laboratories, Ontario; or recombinant epo, Amgen Biologicals, Thousand Oaks, CA), transferred to 25-cm² flasks (Corning Glass Works, Corning, NY) or 24-well microtiter plates (Costar, Cambridge, MA), and cultured at 37°C, 95% humidity, 5% CO₂. Cells were washed free of inoculum virus on day 1. Cells and culture supernatants were harvested from 12 hours to 9 days after inoculation.

Morphology and immunofluorescence. Morphology of infected and control cultures was determined by Wright's-Giemsa staining of cytocentrifuged preparations from periodically sampled cultures. For immunofluorescence, cytocentrifuge preparations were fixed in acetone at -20°C for 30 seconds; washed twice in phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), or gelatin; blotted dry; and stored frozen until staining with rabbit antiserum to B19 capsid proteins.¹³ Optimal results were obtained with anti-B19 antiserum diluted 1:10, 25 μ L/slide $\times$ 30 minutes, followed by two washes in PBS-BSA and application of affinity purified antirabbit IgG-fluorescein isothiocyanate (FITC) (Kierkegaard Perry, Gaithersburg, MD), 1:50, 50 μ L $\times$ 30 minutes. Slides were examined using a Zeiss fluorescent microscope.

Separation of erythroid and nonerythroid cells. For some experiments erythroid and leukocyte fractions were separated by a panning technique. Cells were harvested from culture and washed twice with IMDM-2%FCS. Two $\times 10^7$ cells were incubated in 500 μ L IMDM-10%FCS containing anti-Leu M1 and anti-HLe-1 monoclonal antibodies (MoAbs) (Becton Dickinson, Mountain View, CA), 50 μ L each, one hour at 4°C. The cells were washed three times with IMDM-2%FCS and then added to plastic plates coated with

THE B19 PARVOVIRUS is the etiologic agent of fifth disease, a common rash illness of childhood^{1,2} and a cause of a polyarthralgia syndrome in adults.³⁻⁵ B19 infection in persons with chronic hemolysis leads to abrupt but temporary worsening of anemia, a syndrome termed transient aplastic crisis.⁶⁻⁸ Purified B19 parvovirus has been shown in vitro to inhibit erythroid colony formation by normal human bone marrow cells^{9,10} and demonstrated morphologically within isolated erythroid progenitors.¹¹ While clonal assays have allowed identification of a target of the virus as a human hematopoietic progenitor cell, the small number of cells employed in colony culture systems makes them inadequate for either molecular study of virus replication or for virus propagation. Previous efforts by ourselves and others to grow B19 parvovirus in cultured cell lines have failed.

Recently we reported detection of replicative forms of B19 DNA from the nuclei of human erythroid bone marrow cells infected with B19 parvovirus in vitro and cultured in suspension.¹² Replication of B19 in these cultures, as in clonal assays, was highly dependent on the content of erythroid cells and the presence of the hormone erythropoietin. We report here the effect of B19 infection on erythroid cell number and morphology in human bone marrow cultures; the restriction of B19 replication to erythroid cells; localization of viral replication and encapsidation to nuclei; the time course of B19 production in nuclei, cytosol, and supernatant; and the production of large relative amounts of highly infectious B19 virus at low multiplicity of infection.

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antimouse IgG-IgM for one hour at 37°C. Erythroid cells (>95% by morphology) were recovered in the nonadherent fraction. Leukocytes were recovered by treatment with a solution of 0.05% trypsin-0.02% EDTA (GIBCO Laboratories, Grand Island, NY). For coating of plates, 10 mL of 0.5 mg/mL solution of affinity-purified antimouse IgG-IgM (Cooper Biomedical, West Chester, PA) were added to 100-mm Falcon dishes (Becton Dickinson, Oxnard, CA) and incubated overnight at 4°C. Plates were washed immediately prior to use with IMDM-2%FCS.

Subcellular localization. Cells were washed with Hanks' balanced salt solution (HBSS), suspended in cold reticulocyte standard buffer (RSB; 10 mmol/L NaCl, 15 mmol/L MgCl₂, 10 mmol/L Tris-HCl, pH 7.5)-0.2% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 100 kallikrein inactivator U/mL of aprotinin (Boehringer-Mannheim, Indianapolis), homogenized in a Dounce apparatus (Fisher Scientific, Silver Spring, MD), and separated into cytoplasm and nuclear fractions by centrifugation at 400 × g × ten minutes. The cytoplasm fraction was cleared by centrifugation in a Microfuge apparatus (Beckman, Palo Alto, CA) at 12,000 rpm × five minutes; B19 parvovirus does not sediment under these conditions. Nuclei were rinsed twice in 0.25 mol/L sucrose, 5 mmol/L MgCl₂, 0.2% Triton X-100, 0.1 mmol/L PMSF. Fractionation was confirmed microscopically.

Reinfection experiments. Infectivity of virus in culture supernatants was compared to the infectivity of B19 present in sera from an acutely infected patient. For these experiments primary cultures of erythroid bone marrow were washed free of residual inoculum at day 1, and supernatants were collected for testing two to three days later. Reinfection was accomplished by adding aliquots of supernatants, 20 to 500 µL, or Minor II serum, 0.004 to 0.13 µL, to a total volume of 1 mL containing 2 × 10⁶ normal bone marrow cells in IMDM-20%FCS, 1 U/mL epo. The reinfected cell cultures were incubated for a further two days, the cells were harvested, and input and output B19 DNA was quantitated by dot blot analysis.

Molecular studies. In situ hybridization. In situ hybridization was performed by slight modification of published techniques.¹⁴ In brief, cytocentrifuge preparations were fixed in 4% paraformaldehyde/PBS and stored in 70% ethanol until use. Slides were acetylated in acetic anhydride-triethanolamine and pretreated with a glycine solution to reduce nonspecific binding. The cell preparations were hybridized without prior denaturation using the nick translated ³⁵S-pYT103 probe (derived from a full length cloned B19 DNA¹⁵) in 50% formamide, 2 × SSC, 10% dextran sulfate at 42°C for four to six hours. After washing, hybridized preparations were autoradiographed with NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY) for three days.

DNA extraction. DNA was prepared from whole cells, cytoplasm, nuclei, culture supernatants, and Minor II serum by 0.5% sodium dodecyl sulfate (SDS), 0.1 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, 200 µg/mL proteinase K (Boehringer Mannheim Biochemicals) treatment overnight at 37°C followed by phenol-chloroform extraction. This preparation was used for dot blot analysis. For Southern analysis DNA was further purified by ethanol precipitation, RNase A treatment, 100 µg/mL, proteinase K digestion, repeated phenol-chloroform extraction, and a final ethanol precipitation.

DNA dot blot analysis. NaOH was added to DNA samples from 2 × 10⁵ cells or 200 µL of culture supernatants to 0.3 mol/L and heated to 65°C × 1 hour to destroy RNA and to denature DNA. After neutralization, serial dilutions in 10 × SSC were applied to a minifold dot blot apparatus (Schleicher and Schuell, Keene, NH). After baking at 80°C for two hours, immobilized DNA on nitrocellulose filters (Schleicher and Schuell) was hybridized with ³²P-nick-translated pYT103 insert probe in 6 × SSC, 0.5% SDS, 5 ×

Denhardt's solution, 100 µg/mL denatured salmon sperm DNA at 65°C overnight, washed three times with 2 × SSC-0.1% SDS for five minutes at room temperature, two times with 2 × SSC-1% SDS for 30 minutes at 65°C, two times with 0.1 × SSC-0.1% SDS for 30 minutes at 55°C. Kodak X-Omat AR film was exposed to filters to develop autoradiographs. B19 quantity or B19 genome copy number/cell was determined by comparison to 1 ng B19 DNA from pYT103.

Southern analysis. Parvoviruses are single-stranded DNA viruses, and to perform restriction enzyme analysis, the positive and negative strands, separately packaged within virions, are required to anneal in vitro. B19 DNA from the nuclei, cytoplasm, and supernatants of infected bone marrow cultures was annealed by five-hour incubation at 57°C under high salt conditions (100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA). An appropriate amount of DNA from each fraction, determined by dot blot analysis, was digested with Bam HI. Completion of enzyme digestion was determined by parallel digestion of 0.5 µg lambda DNA with an aliquot of the reaction mixture followed by gel electrophoresis. Undigested (annealed) and Bam HI-digested samples were analyzed by 0.8% agarose gel electrophoresis, Southern transfer, and hybridization with pYT103 probe.

DNase I digestion. B19 parvovirus-infected cells were separated into cytoplasm and nuclear fractions as described above. One × 10⁶ nuclei (about 10 µg DNA) were suspended in 100 µL of reaction buffer (0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaCl, 5 mmol/L MgCl₂, 0.1 mmol/L PMSF). Cytoplasm prepared from 3 × 10⁶ cells was diluted with the same reaction buffer to 100 µL; 10 µg of carrier DNA extracted from uninfected bone marrow cells was added. The particulate fraction of supernatant was concentrated by sedimentation through a cushion of 40% sucrose in PBS at 38,000 rpm for 12 hours at 4°C and suspended in PBS containing 0.02% BSA; the amount equivalent to 1 mL of supernatant with carrier DNA was suspended to a final volume of 100 µL in reaction buffer. DNase I was added to each fraction at final concentrations of 0, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 µg/mL. After ten minutes at 37°C, digestion was terminated by addition of equal volumes of 2× lysis buffer (1% SDS, 20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L EDTA, 1,000 µg/mL proteinase K). DNA was rapidly extracted by phenol-chloroform extraction under low salt conditions to prevent annealing of positive and negative strands. B19 DNA was analyzed by Southern transfer after 1% agarose gel electrophoresis. The efficiency of DNase I digestion was assessed by ethidium bromide staining of gels before Southern transfer. To determine the sensitivity of single-stranded DNA to DNase I in comparison with double-stranded DNA, purified DNA extracted from Minor II serum or from infected nuclei were similarly digested and analyzed.

RESULTS

Effect of B19 on cell growth in suspension cultures. Inoculation with B19 parvovirus of erythroid bone marrow suspension cultures, obtained from patients with sickle cell disease, greatly reduced the number of erythroid cells, as determined morphologically (Fig 1, Table 1). In control cultures treated with equivalent amounts of normal sera, the total cell number increased modestly after about one week of culture, and the absolute number of erythroid cells remained constant. In contrast, B19-infected cultures showed a marked reduction in erythroid cell content beginning at day 2; by day 9 of culture, erythroid cells were consistently <10% of total cells and occasionally as low as 1%. The content of

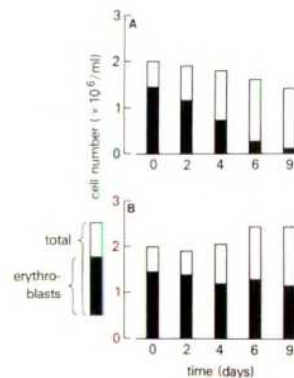


Fig 1. Effect of B19 parvovirus infection on total and erythroid cell number. Cultures were inoculated with serum containing B19 (A) or normal serum (B), $10 \mu\text{L}/5 \times 10^7$ cells, incubated in IMDM-20% FCS, 1 U/mL epo for times shown on the abscissa. Cell number was determined by counting in a hemacytometer and the proportion of erythroblasts by differential counting of Wright's-Giemsa-stained cytocentrifuge preparations.

macrophage and granulocyte cells in culture was unaffected by B19 addition.

The morphological appearance of isolated erythroblasts in B19 infected cultures was frequently very abnormal (Fig 2). Infected cultures were characterized by the presence of cells of very large size, immature chromatin, and absence of hemoglobinization. Some cells showed large eosinophilic, nucleoli-like nuclear inclusion bodies.

Estimate of number of B19 target cells and B19 genome copy number. The proportion of cells infected with B19 parvovirus was estimated by in situ hybridization under conditions of high viral input (see below) 48 hours after inoculation of cultures. Under the conditions of hybridization used, both single-stranded DNA and RNA were detected. Approximately 15% of total cells from infected cultures showed dense accumulation of grains after hybridization with the B19-specific probe (data not shown). In control, uninfected cultures, no cells or very rare cells showed grains (data not shown). When infected bone marrow cultures were separated into erythroid and leukocyte fractions by a pan-

Table 1. Effect of B19 Parvovirus Inoculation on Total and Erythroid Cells in Suspension Bone Marrow Cultures

Experiment	Days in Culture	Cells in Culture ($\times 10^6/\text{mL}$)			
		Infected		Control	
		Total	Erythroid	Total	Erythroid
1	0	2.0	1.4	2.0	1.4
	2	1.9	1.2	1.9	1.4
	4	1.8	0.7	2.1	1.2
	6	1.6	0.2	2.4	1.3
	9	1.4	0.1	2.4	1.2
2	0	2.0	1.0	2.0	1.0
	2	1.7	0.5	1.8	1.0
	4	1.7	0.4	1.9	0.9
	6	1.1	0.1	2.3	1.0
3	0	2.0	1.0	2.0	1.0
	2	2.5	0.9	2.9	1.5
	4	1.6	0.4	3.1	1.2
	5	1.5	0.2	2.8	1.0

Experiments were performed as described in the legend to Fig 1 (the graphic display of experiment 1). Results did not vary with the patient source of bone marrow except for the initial proportion of erythroblasts.

ning method utilizing antileukocyte MoAbs, only erythroid cells demonstrated B19-specific signals by in situ hybridization (Figs 3A, 3B). Between 30% and 40% of erythroid cells were infected. Grains were localized to large and intermediate cells and not to small cells of normoblast size.

To more accurately determine the amount of B19 produced in each target cell, dot blot analysis was performed on each cell fraction (Fig 4). No B19 DNA was detected in uninfected cultures. The erythroid fraction from infected cultures showed a strong B19 DNA signal, which by titration was equivalent to 10,000 genome copies/erythroid cell. Based on the estimated proportion of erythroid cells containing B19 sequences by in situ hybridization, the genome copy number in each infected target cell was approximately 25,000 to 30,000. In fractions enriched by panning for granulocytes, monocytes, and lymphocytes (contaminated <1% with recognizable erythroid cells by morphological

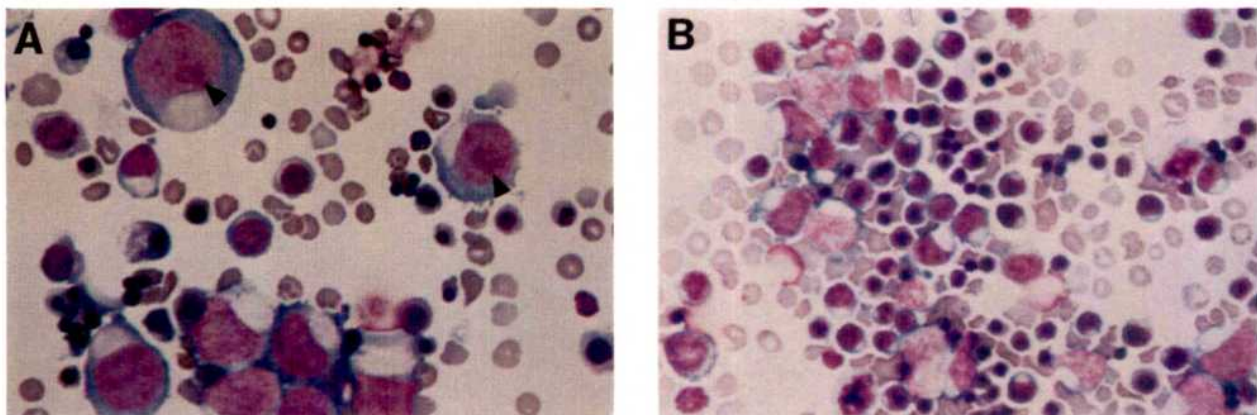


Fig 2. Morphology of B19 infected erythroid cells. Cytocentrifuge preparations from infected (A) and control (B) cultures at 48 hours were stained with Wright's-Giemsa after separation of erythroid cells by panning as described. Some of the large eosinophilic nuclear inclusions are noted by arrowheads. Original magnification $\times 650$; current magnification $\times 455$.

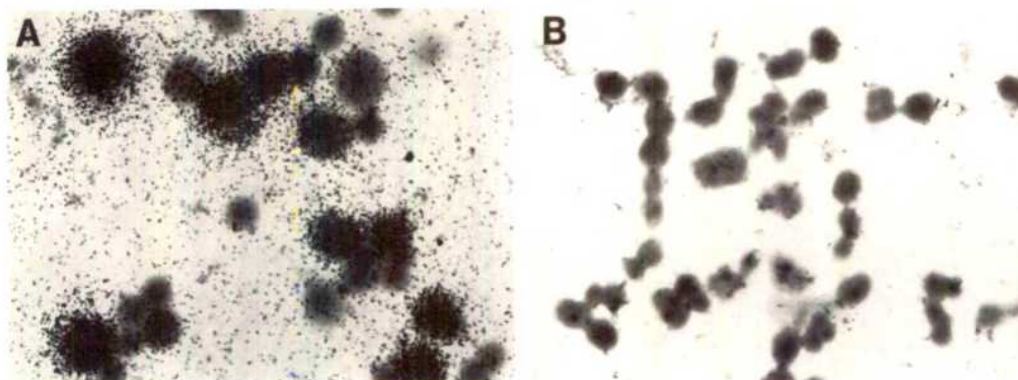


Fig 3. In situ hybridization of B19 infected cells. Cyto centrifuge preparations of separated erythroid (A) and leukocyte (B) fractions from infected cultures obtained at 48 hours were hybridized with ^{35}S -labeled pYT103, a B19 specific molecular probe as described. Erythroid and leukocyte fractions were separated by panning. Original magnification $\times 860$; current magnification $\times 568$.

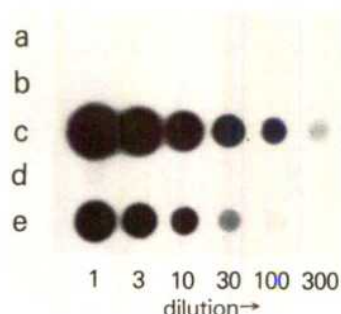


Fig 4. Quantitation of B19 DNA in erythroid and leukocyte bone marrow cells by dot blot analysis. Mononuclear cells from the bone marrow of a patient with sickle cell disease were infected with Minor II serum as described in the legend to Fig 1. Forty-eight hours after inoculation cells were harvested and separated by panning into erythroid and leukocyte fractions. B19 DNA was quantitated by serial dilutions in a dot blot assay of 10^5 cells: (a) uninfected erythroblast fraction; (b) uninfected leukocyte fraction; (c) B19 infected erythroblast fraction; (d) B19 infected leukocyte fraction; (e) pYT103, 10^3 pg and serial dilutions.

criteria), the signal was approximately 1/1,000 that of erythroid fractions.

A similar proportion of total bone marrow cells were infected as determined by immunofluorescence with a B19 capsid-specific antiserum (Fig 5). Staining was predominantly cytoplasmic, and specific staining was detected in 20% to 30% of cells of infected cultures at 48 hours.

B19 replication and encapsidation. DNA was extracted from isolated nuclei, cytoplasm, and supernatant of infected cultures at 48 hours and analyzed for the presence of characteristic replicative intermediate forms.¹² As shown in Fig 6, DNA from nuclei but not from cytoplasm or supernatants showed doublets of Bam HI restriction enzyme-digested fragments (3.9/3.8, 1.5/1.4 kb), generated from extended and turn-around forms of the replicative intermediates. Confirmatory results were obtained with DNA extracted under low-salt conditions to prevent annealing of the separately packaged positive and negative strands.¹²

To determine the site of encapsidation of B19 virus, DNase I sensitivity was examined for B19 DNA from isolated nuclei as well as the cytoplasm and supernatant of

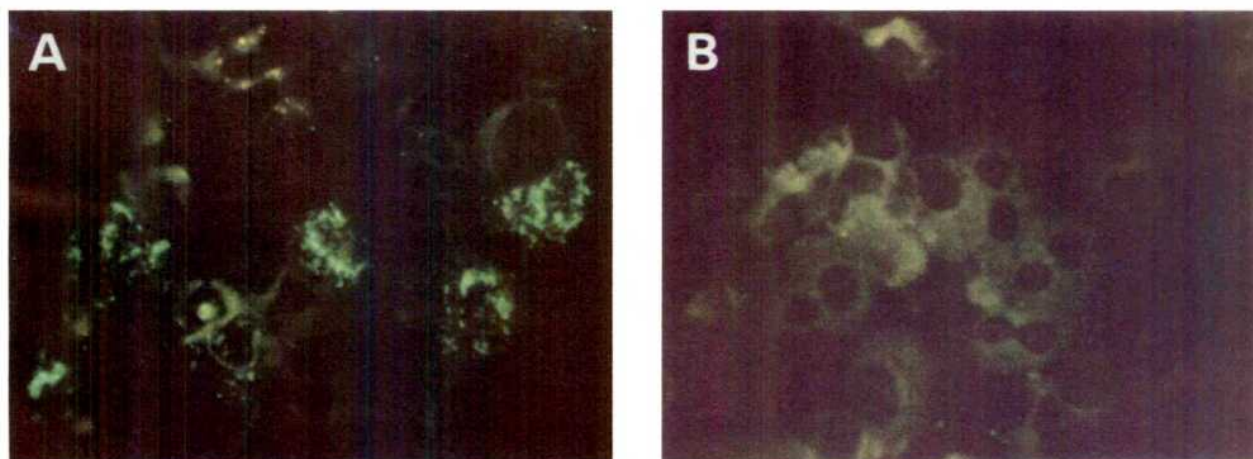


Fig 5. Immunofluorescence of B19 protein in infected cells. Cyto centrifuge preparations obtained 48 hours after B19 inoculation were fixed in acetone and stained with rabbit anti-B19 capsid protein antiserum followed by antirabbit IgG-FITC: (A) infected, (B) control. Original magnification $\times 840$; current magnification $\times 664$.

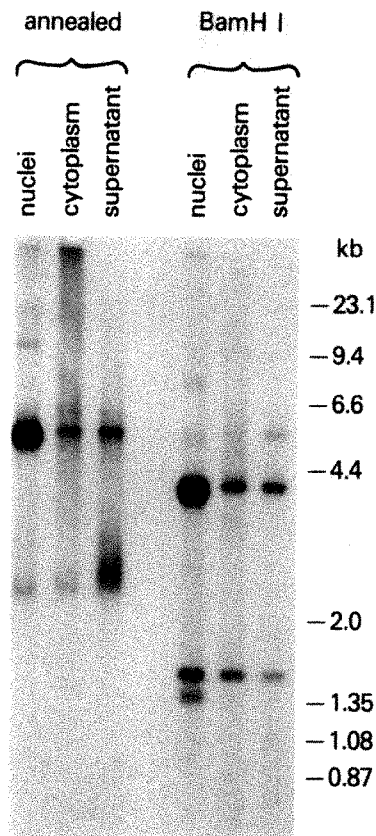


Fig 6. Restriction of B19 replication to nuclei. Undigested (annealed) and Bam HI-digested DNA samples from isolated nuclei, cytoplasm, and supernatants of B19-infected cultures were subjected to Southern analysis.

infected cultures (Fig 7). In this method, nuclei, cytoplasm, and supernatants were digested with DNase I prior to DNA extraction (intact virions are resistant to DNases¹⁰). The double-stranded monomer replicative form in nuclei was DNase I sensitive, but single-stranded virus DNA in nuclei was completely resistant to enzyme, similar to virus DNA in cytoplasm and supernatant. Purified single-stranded DNA was equally well digested as double-stranded DNA by DNase I, whether extracted from virus present in serum or from infected cell nuclei (Fig 8). This combination of results implied that viral encapsidation occurred within the nucleus immediately after the cleavage of double-stranded replicative forms to single-stranded forms.

Time course and subcellular localization of B19 production. B19 production was estimated using dot blot analysis

of DNA in infected cells and supernatants (Fig 9A). Ten microliters of serum containing virus was added to a total of 5×10^7 erythroid bone marrow cells. The total amount of B19 DNA in cultures (cells and supernatants) was initially reduced at 12 hours, suggesting partial degradation of the input virus. The first strong signal of B19 DNA within cells appeared at 18 hours, and B19 DNA content in cells thereafter increased dramatically, peaking at 48 hours. The decline in B19 DNA within cells from days 2 to 9 paralleled the fall in erythroid cell number. For measurements of B19 DNA in supernatant, residual viral inoculum was removed by washing at day 1. B19 DNA appeared in the supernatants of infected cultures at 32 hours and reached a plateau at day 4.

The subcellular localization of B19 DNA was determined by dot blot analysis after fractionation of infected cells into cytoplasmic and nuclear compartments (Fig 9B). About 95% of B19 DNA was localized to the nucleus, and this ratio remained stable throughout the culture period.

Effect of multiplicity of infection on B19 virus production. Efficiency of virus production was highly dependent on the ratio of input virus to bone marrow cells (Fig 10). When measured at day 2, relatively high concentrations of serum-containing virus were required for maximal output. However, when measured at days 5 or 9, tenfold less virus resulted in maximal B19 production, measured in total cultures, cells, and supernatants. At low amounts of input virus, about 50 $\times$ higher amounts of B19 were produced in supernatant and >200 $\times$ output virus was measured in total cultures (Fig 10B). These results suggest amplification of the virus signal at low input concentrations by infection of target cells with newly synthesized virus.

Reinfection with culture supernatants. To determine if infectious virus was produced in erythroid bone marrow cultures, aliquots of supernatants from days 3 to 4 were added directly to fresh bone marrow cultures (Table 2). Production of B19 in the secondarily infected cultures was assayed at day 2 and compared with B19 production by simultaneous cultures infected with equivalent amounts of B19-containing serum. In supernatants from two cultures, relative infectivity was superior to serum, and in another the supernatant was equally potent to serum.

DISCUSSION

The suspension culture system for B19 parvovirus has several important features. First, virus replication is dependent on the erythroid cell content of the cultures and the

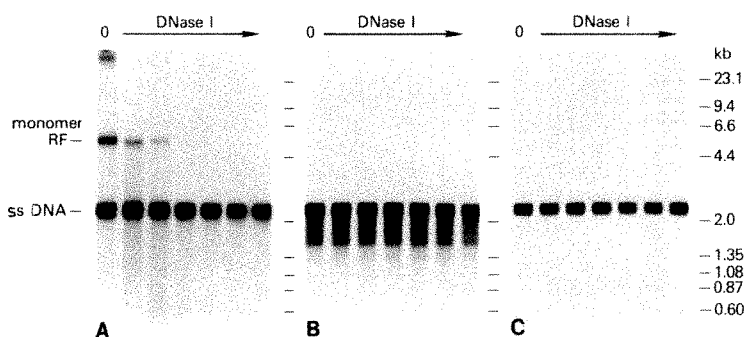


Fig 7. Restriction of B19 encapsidation to nuclei. Nuclei (A), cytoplasm (B), and supernatant (C) of infected cultures were digested with DNase I at increasing concentrations of enzyme as described in the Methods, and B19 DNA extracted under low salt conditions was subjected to Southern analysis.

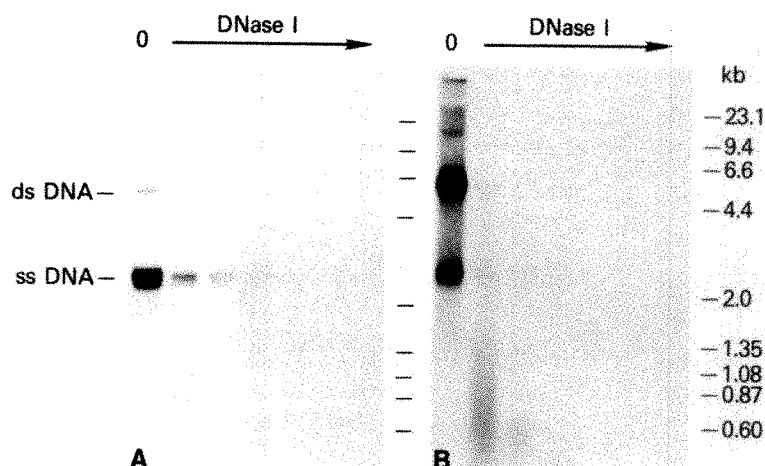


Fig 8. Comparison of DNase I sensitivity of single and double-stranded B19 DNA. Purified DNA extracted from Minor II serum (virions; [A]) and from infected nuclei (B) were digested as described in the legend to Fig 7.

presence of erythropoietin; virus replication does not occur in bone marrow cultures lacking detectable erythroid progenitors¹² or in the leukocyte fraction of bone marrow. Second, B19 virus is released from cells into culture supernatants, as demonstrated by the appropriate banding on CsCl₂ gradients of ³H-thymidine-labeled parvovirus particles,¹² the 50-fold output of virus into supernatant at low multiplicity of infection, and the potency of supernatants in reinfecting bone marrow. Because the highest ratio of virus output to input occurred at low multiplicity of infection (moi) characteristic of cytolytic infection, the usefulness of this culture system for production of virus is limited by the number of target cells that can be obtained in a bone marrow aspirate and the

difficulty of maintaining erythropoiesis in vitro for extended periods of time.

Except for the use of terminally differentiating cells as targets, B19 propagation in marrow cells appears quite biologically similar to the propagation of other autonomous parvoviruses in cell culture lines. Animal parvoviruses have generally been cultivated in monolayer cell cultures and quantitated with plaque forming assays,¹⁶ complicating comparison with the bone marrow culture system for B19. All autonomous parvoviruses require S phase cells for replication.¹⁷ The kinetics of infection of B19 parvovirus appear similar to that of other parvoviruses, with appearance of

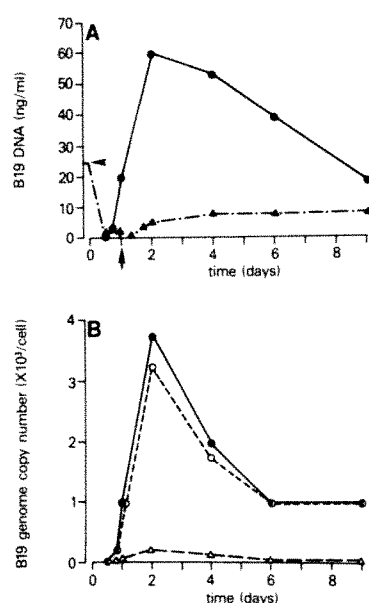


Fig 9. Time course and subcellular localization of B19 production. (A) Bone marrow cultures were inoculated as described in the legend to Fig 1 at time 0; the level of input virus is noted (arrowhead). Cells in culture were washed free of residual inoculum at day 1 (arrow). B19 DNA was quantitated by dot blot analysis in reference to pYT103 for total cells (●—●) and culture supernatants (▲—▲). Data are expressed as ng B19 DNA present in 1 mL of culture (cells or supernatant). (B) B19 DNA was quantitated in total cells (●—●), nuclei (○—○), and cytoplasm (△—△) fractions obtained after Dounce homogenization. Data are expressed as B19 genome copy number/cell.

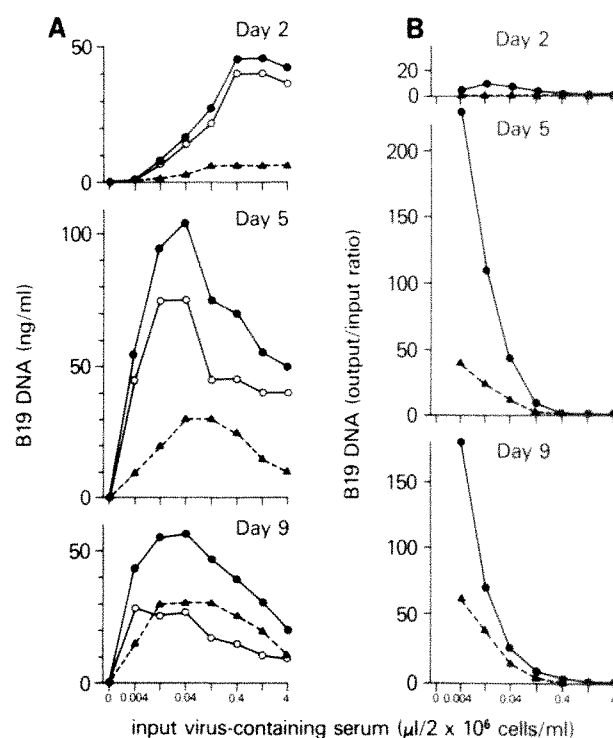


Fig 10. Effect of multiplicity of infection on B19 virus production. Cultures of erythroid bone marrow were performed as described, but the amount of input virus, B19-containing serum, was varied as shown. B19 was quantitated by dot blot in total cultures (●—●), cells (○—○), and supernatants (▲—▲). (A) B19 DNA, ng/mL culture; (B) B19 DNA, output/input ratio.

Table 2. Relative Infectivity of B19 in Culture Supernatants

Experiment No.	Output B19 DNA (Genome Copy No./Cell)	
	Supernatant	Serum
1	1,500	450
2	1,000	400
3	450	400

Primary cultures of erythroid bone marrow were washed free of residual inoculum at day 1, and supernatants were collected for testing two to three days later. In each experiment a separate supernatant was compared with Minor II serum for ability to infect normal bone marrow. The quantity of B19 DNA produced in secondary cultures at 48 hours was measured by dot blot analysis. Shown are the results with input B19 DNA equivalent to 1.5 ng/10⁶ bone marrow mononuclear cells, as determined by dot blot analysis; this figure corresponds to the linear region of the multiplicity of infection curve for harvesting of virus at 48 hours (Fig 10A, day 2). In experiments 2 and 3, supernatants were tested over a wide range of input amount (0.04 to 1.5 ng/10⁶ cells); the data shown represent optimal output with unconcentrated supernatants.

virus in cells about 12 hours after inoculation as for rodent viruses,¹⁸⁻²⁰ feline panleukopenia virus,²¹ and bovine parvovirus²²; however, virus quantity rapidly increases in regenerative cell culture systems. Most virus produced remains cell associated for B19 in bone marrow and for other parvoviruses, and the appearance of virus in culture supernatants is delayed compared with cell-associated virus.^{19,21,22} Virus replicates and is encapsidated only within the nucleus, consistent with electron microscopic data.¹¹ As shown in the cell fractionation experiments, most of the B19 DNA produced remained in nuclei rather than entering the cytosol for eventual release into the supernatant. In contrast to B19 DNA, most B19 protein was localized to the cytoplasm. Immunofluorescent staining with anticapsid antiserum of isolated immature bursts¹¹ as well as cells from these suspension cultures showed localization of B19 capsid proteins in the cytoplasm.

Estimates of true multiplicity of infection are complicated by the absence of a convenient assay for infectious B19 particles, such as a plaque titration, and uncertainty concerning the nature and number of parvovirus target cells in erythroid bone marrow. Assuming roughly 90% of virus input is infectious (by subtraction of the percentage of empty capsids usually seen in immune electron microscopy), a viral titer of 2×10^{13} particles/mL serum, and 15% of the bone marrow cells serving as targets, the optimal multiplicity of infection at 48 hours was about 20,000 viral particles/cell

and at 120 hours, 2,000 viral particles/cell. However, the true optimal moi of B19 may be considerably lower if the ratio of infective particles to particles is as low as directly measured for other parvoviruses (minute virus of mouse, 1:300 to 400¹⁸; adenoassociated virus, 1:10 to 100¹⁶).

B19 parvovirus infection greatly reduces the number of recognizable erythroid cells in suspension cultures and alters the morphology of residual erythroid precursors; this culture system thus reproduces two of the cardinal features of the bone marrow of patients with transient aplastic crisis,^{22,23} characterized by a paucity of erythroid precursors and the presence of giant pronormoblasts, the latter possibly related to arrest of mitosis.²¹ Similar bone marrow morphology has been seen in a case of persistent B19 parvovirus infection resulting in chronic bone marrow failure.²⁴ To date human bone marrow cells are the only cells identified to support B19 parvovirus propagation in vitro. In normal volunteer infections, viremia and reticulocytopenia were coincident early in the clinical illness.²⁵ The symptoms of fifth disease—rash and polyarthralgia—are late manifestations at the time of appearance of IgM and IgG antibodies specific for B19; these symptoms do not appear with great frequency in transient aplastic crisis patients. These clinical differences may result from different quantities of B19 virus and antibody to virus produced in hematologically normal individuals compared with patients with chronic hemolysis who have greatly expanded erythroid cell compartments.

Because of the much larger numbers of cells that can be studied from suspension cultures compared with clonal assays, a fuller description of the interaction of B19 and hematopoietic progenitors at the cellular and molecular levels is now possible. This cell culture system has allowed construction of a transcriptional map²⁶ and definition of the structure and function of capsid and noncapsid B19 proteins.²⁷ Perhaps most important, the availability of a suitable culture system with readily assayed variables of infection—cell number, morphological changes, and B19 gene sequences by dot blotting—in conjunction with nonpermissive cells in culture should allow characterization of the basis of B19 parvovirus' extraordinary specificity for erythroid cells.

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Homosexual Men With Thrombocytopenia Have Impaired Reticuloendothelial System Fc Receptor-Specific Clearance

By Bradley S. Bender, Thomas C. Quinn, and Jerry L. Spivak

Classic immune thrombocytopenia purpura (ITP) occurs predominantly in women and is associated with either normal or impaired Fc receptor-mediated clearance of antibody-coated cells. Recently, an increasing incidence of thrombocytopenia has been observed in homosexual men, but whether Fc receptor-mediated clearance of antibody-coated cells is normal or impaired in these men is unknown. To study this question, we measured the *in vivo* clearance of anti-Rh₀(D) IgG antibody-sensitized ⁵¹Cr-labeled autologous red cells in five homosexual men with thrombocytopenia without an evident cause. All five had antibodies to human immunodeficiency virus, and four had circulating immune complexes as determined by a C1q-binding assay. Two of the men tested also had an increase in platelet-associated IgG. In the four homosexual men with platelet counts of 20,000/μL or less, the clearance half-time of IgG-sensitized red cells was prolonged (mean, 106 minutes;

range, 72 to 140 minutes) as compared with the clearance of such cells in five hematologically normal men (mean, 39 minutes; range 30 to 50 minutes; *P* < .005). One homosexual man with a platelet count of 81,000/μL had a normal clearance half-time (30 minutes). Three patients whose platelet counts increased after corticosteroid therapy were restudied. In all three, the clearance of antibody-coated cells was shortened and returned to normal in the one patient who had achieved a complete remission. No correlation was observed between the presence of platelet-associated IgG or circulating immune complexes and the clearance half-time. These data indicate that severe thrombocytopenia occurring in homosexual men as in some patients with classic ITP is associated with defective *in vivo* Fc receptor-mediated clearance of antibody-coated cells.

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INFECTION with the newly described retrovirus human immunodeficiency virus (HIV) leads to a variety of consequences ranging from asymptomatic carriage to the devastating acquired immunodeficiency syndrome (AIDS).¹ Other illnesses such as unexplained lymphadenopathy, idiopathic neuropathies, persistent diarrhea, fever, weight loss, and oral thrush have also been linked both epidemiologically and serologically to infection with HIV.¹

Recent reports indicate that the occurrence of thrombocytopenia is increasing among homosexual men,^{2,3} patients with hemophilia,⁴ and intravenous drug users⁵—the same groups at highest risk of developing AIDS.⁶ Whether the mechanism for thrombocytopenia seen in HIV-positive patients is similar to that for classic immune thrombocytopenia purpura (ITP) is unknown. Classic ITP occurs primarily in women⁷ and is associated with either normal or impaired splenic Fc receptor function.^{8,9} Since Fc receptor function is impaired in patients with AIDS,^{10,11} it has been postulated that homosexual men with ITP may also have defective Fc receptor function.^{5,12,13} To examine this, we studied the rate of clearance from the circulation of ⁵¹Cr-labeled, IgG-sensitized autologous erythrocytes in homosexual men with thrombocytopenia before and after therapy.

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MATERIALS AND METHODS

Subjects. The subjects were five homosexual men with thrombocytopenia who were otherwise healthy. None of them had recent weight loss, fever, unexplained lymphadenopathy, or any infections. All were HIV antibody-positive by both enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Their clinical and laboratory features are given in Table 1. As controls, five hematologically normal, healthy, age-matched heterosexual men who were HIV-seronegative were studied. Informed, written consent was obtained from all subjects.

Clearance studies. Clearance studies were performed as previously described.¹⁴ In brief, the subjects' erythrocytes were collected, washed with normal saline, and labeled with ⁵¹Cr (ICN Pharmaceuticals, Irvine, CA) for 30 minutes at 37°C in a shaking water bath. An aliquot of the ⁵¹Cr-labeled cells was sensitized by the dropwise addition of purified IgG-anti-Rh₀(D) followed by a 30-minute incubation at 37°C. After two saline washes, a portion of the cells was injected into a forearm vein. Timed, serial bleedings were performed, and erythrocyte survival was calculated from the clearance half-time of the labeled red cells and defined as the time at which 50% of the labeled cells had been removed from the circulation.

Other studies. The presence of circulating immune complexes was detected by a C1q-binding assay.¹⁵ Antiplatelet IgG antibody was assayed by a radiolabeled Coombs' antiglobulin test.¹⁶ Serum antibody to HIV (ELISA assay) was measured with a commercial kit (Litton Bionetics, Charleston, SC) and confirmed by Western blotting.

RESULTS

Clearance studies. Infusion of IgG-sensitized erythrocytes is normally followed by progressive splenic sequestration and destruction.¹⁷ In five hematologically normal and HIV-seronegative men, the mean clearance half-time of IgG-sensitized erythrocytes was 39 minutes (range, 30 to 50 minutes). We have previously determined that Fc receptor-specific clearance is identical in heterosexual and homosexual individuals.¹⁰ Furthermore, only two of nine patients with an AIDS-related illness had a clearance that was outside the normal range. Thus, HIV infection alone does not lead to a defect in Fc receptor-mediated clearance function.¹⁰

Table 1. Clinical Data on HIV-Positive Patients With Thrombocytopenia

Pt	Age	HIV Antibodies	Pretreatment					Therapy	Posttreatment				
			Platelet Count (μL)	Platelet-Associated IgG*		Immune Complexes†	Fc Receptor Clearance Half-time‡ (min)		Platelet Count (μL)	Platelet-Associated IgG		Immune Complexes	Fc Receptor Clearance Half-time (min)
				Direct	Indirect					Direct	Indirect		
1	27	+	8,000	<1.5	<2.0	48%	125	Cs	188,000	10	<2.0	5.0%	30
2	32	+	13,000	6.8	<2.0	15%	72	Cs, SPLX	197,000	ND	ND	ND	ND
3	42	+	81,000	7.9	11.0	66%	30	None	ND	ND	ND	ND	ND
4	50	+	20,000	<1.5	<2.0	40%	140	Cs	105,000	<1.5	<2.0	6.1%	120
5	41	+	19,000	ND	ND	ND	87	Cs	31,000	14.1	<2.0	4.3%	77
Controls		—	>150,000	<1.5	<2.0	<10%	30–50						

Abbreviations: Pt, patient; ND, not determined; Cs, corticosteroids; SPLX, splenectomy.

*Expressed as femtograms per platelet.

†Expressed as the percentage of protein-bound ^{125} I-C1q precipitated from serum.

‡Clearance studies were performed before therapy was initiated in all subjects except patient 4.

Figure 1 depicts the clearance curves of the five homosexual patients with thrombocytopenia. In the four men with platelet counts $\leq 20,000/\mu\text{L}$, the clearance half-time was significantly prolonged (mean, 106 minutes; range, 72 to 140 minutes; $P < .005$). The one homosexual man with a platelet count of $81,000/\mu\text{L}$ had a normal clearance. Since reticuloendothelial Fc receptor function can be blocked by circulating immune complexes,¹⁷ the presence of such complexes was examined by a C1q-binding assay. All four patients tested had circulating immune complexes (Table 1), but the greatest amount was present in patient 3 who had the highest platelet count and a normal clearance.

Follow-up studies. Three of the patients (1, 4, and 5) responded to oral administration of corticosteroids with a sustained rise in the platelet count, and one patient (2) required splenectomy for correction of thrombocytopenia; patient 3 has not required any therapy. In follow-up ranging 6 months to 4 years, no patient has developed AIDS or other clinical complications of HIV.

Repeat clearance studies were performed in the three patients who responded to corticosteroid therapy alone. None was receiving therapy at the time of the repeat study. As

shown in Table 1 and Fig 2, the clearance half-time returned to normal in patient 1 who achieved a complete remission in spite of the presence of platelet-associated IgG. Patient 4 who had no demonstrable platelet-associated IgG had an improvement in clearance half-time, whereas the clearance half-time of patient 5 who had the least response to corticosteroids was essentially unchanged.

DISCUSSION

Specific membrane receptors on phagocytic cells have been demonstrated to be of functional importance in the recognition, attachment, and ingestion of particulate antigens by the reticuloendothelial system.¹⁷ Recent studies have shown that the functional capacity of reticuloendothelial cells in patients with a variety of immunologic illnesses can be assessed by measuring the in vivo clearance of IgG-sensitized autologous erythrocytes.^{8-11,14,17} We have previously reported that splenic Fc receptor-specific clearance is abnormal in patients with AIDS^{10,11} but not in asymptomatic HIV-positive homosexual men or patients with the AIDS-

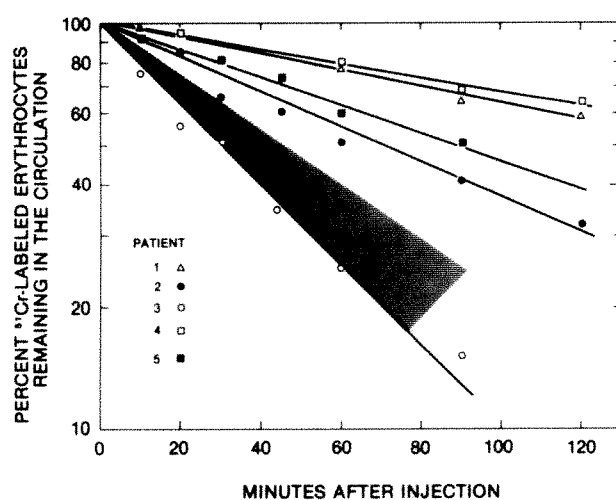


Fig 1. Survival of IgG-sensitized ^{51}Cr -labeled autologous erythrocytes. The shaded area is the range of five healthy volunteers, and the individual clearance curves are from the five homosexual patients with thrombocytopenia.

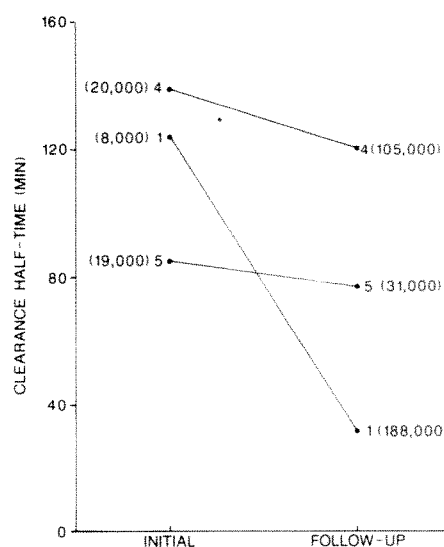


Fig 2. Initial and follow-up Fc receptor clearance half-times in patients 1, 4, and 5. The numbers in parentheses are the patients' blood platelet counts in cells/ μL at the time of each clearance study.

related complex, eg, lymphadenopathy, fever, or weight loss.¹⁰ In this study, we demonstrate that severe thrombocytopenia occurring in HIV-positive homosexual men is also associated with prolonged Fc receptor-mediated clearance. In this regard, the pathophysiology of thrombocytopenia in homosexual men appears similar to some patients with classic ITP.⁹ For example, Kelton et al found normal clearances in five patients with ITP but impaired clearance in three others.⁹ As in our study, the patients with impaired Fc receptor clearance tended to have the most severe thrombocytopenia.⁹ HIV-positive individuals with thrombocytopenia do, however, differ from patients with classic ITP with respect to the presence of immune complexes and a greater degree of platelet-associated IgG.¹²

An impairment of Fc receptor-mediated clearance in the presence of immune-mediated platelet destruction appears paradoxical, and precisely why some patients with classic ITP have a prolonged clearance of IgG-sensitized red cells is unknown. However, some explanations based on the known mechanisms of immune clearance can be offered. First, in some patients with ITP, the liver may be a major site of platelet sequestration,¹⁸ and splenic clearance studies using lightly IgG sensitized¹⁷ ⁵¹Cr-labeled erythrocytes might not accurately reflect the role of the liver. Second, efficient clearance of antibody-coated erythrocytes depends on an intact splenic microcirculation. For example, in rodent malaria, splenic trapping of parasitized erythrocytes induces alterations in the splenic cord microcirculation, thereby causing a delay in clearance function.¹⁹ It is conceivable that trapped, aggregated platelets could also alter the splenic microcirculation, but we are not aware of any experimental evidence to support this contention. Third, phagocytosis is initiated by attachment of IgG-sensitized cells to the macrophage's Fc receptor. It has been suggested that there may be a competition for splenic macrophage Fc receptors between the infused IgG-sensitized red cells and the patient's intrinsically sensitized platelets,⁹ particularly if these cells are sensitized with IgM.²⁰ The treated red cells used in studies such as ours were only lightly coated by IgG, and there may be more efficient or preferential phagocytosis of the patient's IgG- or IgM-coated platelets, thus creating an apparent Fc receptor blockade. Fourth, circulating immune complexes could cause reticuloendothelial blockade,¹⁷ but these are not

usually present in classic ITP. Finally, since in some patients with ITP thrombocytopenia is a consequence of impaired platelet production rather than increased platelet destruction,^{21,22} it is possible that impaired Fc receptor-mediated clearance is another disease-related abnormality but not itself responsible for the thrombocytopenia.

Our observations in homosexual men with ITP provide some insight into these issues. First, it is unlikely that enhanced platelet sequestration by the liver was responsible for thrombocytopenia in our patients since their thrombocytopenia was corrected by either corticosteroids or splenectomy. Second, competition with antibody-coated platelets seems unlikely to explain the impaired clearance of sensitized red cells since there was no correlation between the presence or absence of platelet-associated IgG and impaired Fc receptor-mediated clearance. This was true for immune complexes as well; these are not usually present in classic ITP where Fc receptor-mediated clearance can also be impaired. The possibility that impaired Fc receptor-mediated clearance in homosexual men was due to macrophage infection with HIV²³ is also unlikely since in this situation improvement in clearance by corticosteroid therapy or splenectomy would not be expected. The reciprocal relationship between platelet count and Fc receptor-mediated clearance strongly suggests that these are related either as independent manifestations of the same underlying process or as interrelated processes with preferential sequestration of platelets by macrophages. Whether there is a defect in platelet production in homosexual ITP as has been demonstrated in some patients with classic ITP is unknown.^{21,22}

Even though none of our five patients has progressed to AIDS or related conditions, follow-up studies on larger numbers of such patients with ITP over a longer period of time have revealed that AIDS developed in some.^{3,24} Isolated thrombocytopenia may thus represent a particular immunologic consequence of HIV infection, but the potential for progression to AIDS and the occurrence of impaired Fc receptor function in both situations indicates a strong interrelationship.

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High Serum Levels of Soluble Interleukin 2 Receptor in Patients With B Chronic Lymphocytic Leukemia

By G. Semenzato, R. Foa, C. Agostini, R. Zambello, L. Trentin, F. Vinante, F. Benedetti, M. Chilosi, and G. Pizzolo

By using an enzyme-linked immunosorbent assay, the presence of the soluble form of the interleukin-2 receptor (sIL-2R) was evaluated in the peripheral blood of 54 patients with B cell chronic lymphocytic leukemia (B-CLL). Serum levels of sIL-2R were correlated with clinical features, relevant hematologic and immunologic data, and in some cases, with in vitro functional studies. In 51 patients (94.4%), the levels of sIL-2R were increased as compared with normal age-matched controls ($1,781 \text{ U/mL} \pm 231$ v $276 \text{ U/mL} \pm 26$, respectively; $P < .001$). Although this increase was observed in all stages of the disease and independently of several hematologic and immunologic parameters, a trend toward lower levels of sIL-2R was documented in patients with a less-invasive disease. When

the values were correlated with the functional status of the residual T cell population, it was found that patients with the lowest levels of sIL-2R showed the best mitogenic response and helper capacity. It is suggested that in B-CLL patients the high levels of serum sIL-2R, capable of binding to its ligand, may block the T cell-produced IL-2, thus contributing toward a defective physiological action by this lymphokine. In turn, this defective availability of IL-2 may play a part in the abnormal immunoregulation that is implicated in the hypogammaglobulinemia, susceptibility to infections, and incidence of second neoplasias often observed in this disease.

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B CELL CHRONIC LYMPHOCYTIC leukemia (B-CLL) is a lymphoproliferative disorder characterized by the accumulation of relatively mature monoclonal B lymphocytes. Although the residual T cell population in this disease does not belong to the neoplastic clone, as indicated by the lack of chromosome abnormalities and by the heterozygosity for glucose-6-phosphate dehydrogenase,^{1,2} it has been shown to exhibit a number of abnormalities. These include an increased absolute number,^{3,4} a reversed CD4/CD8 ratio^{5,6} that often correlates with the stage of the disease,⁷⁻¹⁰ an abnormal T cell subset redistribution between peripheral blood (PB) and bone marrow,¹¹ and several functional impairments. The latter include a defective proliferative response to mitogens and allogeneic cells, a reduced colony formation, and a depressed helper and cytotoxic activity.⁷ Although most of the studies point toward multiple functional defects, the precise origin and significance of these T cell abnormalities in B-CLL is unexplained. It is still under discussion whether they represent a primary or a secondary event.

The receptor for interleukin-2 (IL-2R), which is defined by the CD25-related monoclonal antibodies (MoAbs),¹² is present on the cell membrane of T lymphocytes, B lympho-

cytes, and macrophages when these cells are activated or when they belong to a discrete stage of their ontogeny.^{13,14} In B-CLL patients, both residual T lymphocytes and neoplastic B cells have been shown to be capable of expressing IL-2R.^{15,16} It has been recently demonstrated that, in particular in vitro and in vivo conditions,^{17,18} IL-2R may be released from the cell surface in a soluble form (sIL-2R). High serum levels of sIL-2R have been demonstrated in several diseases including hairy cell leukemia and Hodgkin's disease.¹⁹⁻²¹

In view of the fact that IL-2R may be detected on the surface of neoplastic B cells²²⁻²⁴ and that exogenous IL-2 may play a direct role in the proliferation of normal and leukemic B lymphocytes,^{7,15,25} it is tempting to hypothesize that, in some pathological conditions such as B-CLL, alterations of the normal levels of sIL-2R may affect the balance of the autocrine system represented by IL-2 and IL-2R molecules. In turn, this may downmodulate the immune response and lead to defective immunoregulation, which may help to explain the T cell abnormalities reported in B-CLL.⁷ To test this possibility we evaluated the presence of sIL-2R in 54 patients with B-CLL. In all of them the levels of sIL-2R were correlated with the clinical stage, the relevant hematologic and immunologic data, and in some cases, with functional in vitro studies.

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MATERIALS AND METHODS

Patients. The study included 54 patients with B-CLL, 32 men and 22 women, with a mean age of 66 years. The diagnosis was made on the basis of established hematologic criteria and confirmed in all cases by phenotypic analyses on PB cell suspensions. Patients were graded according to Rai's staging system²⁶ as follows: 7 stage 0, 13 stage I, 17 stage II, 9 stage III, and 8 stage IV. The total lymphocyte count ranged between $16 \times 10^9/\text{L}$ and $240 \times 10^9/\text{L}$. Thirty-seven patients never received treatment, and 17 patients had been previously treated with low doses of alkylating agents, but at the time of this study they had not been treated for at least 16 weeks. The mean serum immunoglobulin levels, as determined by radial immunodiffusion, were IgG, $904 \text{ mg/mL} \pm 141$ (control range, 800 to 1,680 mg/mL); IgA, $102 \text{ mg/mL} \pm 27$ (control range, 60 to 400 mg/mL); IgM, $52 \text{ mg/mL} \pm 13$ (control range, 50 to 250 mg/mL).

Twenty-five healthy volunteers in the same age range were used as controls.

Detection of sIL-2R. sIL-2R levels in the serum of B-CLL

patients and of normal controls were determined by using a sandwich enzyme immunoassay (Cellfree, T Cell Sciences, Inc, Cambridge, MA) as described by Rubin et al.¹⁷ Briefly, the IL-2R available in the test samples or in the standards binds to the polystyrene microtiter wells previously incubated with 100 μ L anti-Tac equivalent MoAb (1 μ g/mL). A horseradish peroxidase-conjugated anti-IL-2R MoAb directed against a second epitope on the IL-2R molecule binds to the IL-2 captured by the first antibody and completes the sandwich. After washing to remove the unbound enzyme-conjugated anti-IL-2R MoAb, a substrate solution is added to the wells. The reaction is then stopped and the adsorbance determined at 490 nm. A standard curve is prepared from four IL-2R standards. The IL-2R standard was the cell-free supernatant obtained from phytohemagglutinin (PHA) in vitro-stimulated T cells and was assigned a value of 1,000 IL-2R U/mL.

Lymphocyte separation and T cell purification. Mononuclear cells were separated by a Ficoll-Hypaque (F/H) density gradient from freshly drawn heparinized PB and washed three times in sterile phosphate-buffered saline. Adherent cells were removed after incubation at 37°C for 45 minutes in plastic Petri dishes.

T lymphocytes were purified from non-T cells by rosetting with neuraminidase (Sigma Chemical Co, St Louis)-treated sheep RBC (nSRBC) followed by repeated F/H gradient separations as previously described.²⁷ T cells were recovered after SRBC lysis with 0.83% ammonium chloride and resuspended in RPMI 1640 (GIBCO, Grand Island, NY) at a concentration of 8×10^6 /mL. The purified fractions always contained more than 97% and 90% E-positive cells (in controls and B-CLL groups, respectively), whereas the contamination with surface immunoglobulin-positive cells never exceeded 2%. The viability, assessed by trypan blue exclusion, was always greater than 95%.

Surface marker analysis. The following MoAbs, designated according to the Boston workshop,¹² were used to characterize the phenotype of the enriched T cell populations. OKT3 (CD3) MoAb (Ortho Pharmaceutical Corp, Raritan NJ) defines T cells. OKT4 (CD4)-positive cells include helper/inducer lymphocytes, and cells reactive with OKT8 (CD8) MoAb include cytotoxic/suppressor cells.¹¹ In some cases the CD4/CD8 ratio was also determined directly by double staining using reagents labeled with the fluorochromes phycoerythrin and fluorescein as previously described.¹¹ Similar results were consistently found.

Response to mitogens and helper function. The response of enriched T cells to PHA was evaluated as already reported.²⁸ Briefly, triplicate cultures of 2×10^5 T cells were performed in 200 μ L RPMI 1640 medium (GIBCO) supplemented with 20% fetal calf serum (Microbiological Associates, Walkersville, MA), 2 nmol glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and graded concentrations of PHA-M (Difco, Detroit) ranging from 0.5 to 6 μ g/mL. Cultures were incubated at 37°C in a 5% CO₂ atmosphere for 72 hours. One microcurie of ³H-thymidine (CEA IRE, Sorin Biomedica, Saluggia, Italy; specific activity, 26 mCi/mmol/L) was added over the last six hours of culture and the incorporation measured in counts per minute with a liquid scintillation counter. The results are expressed as the mean cpm/ 10^5 lymphocytes of triplicate cultures at the optimal dose (1.5 μ g/mL).

The effect of total enriched populations on the in vitro pokeweed mitogen (PWM)-driven B cell differentiation was evaluated in a mixture of T cells depleted of cells bearing Fc receptors for immunoglobulin G (T_G) and normal B cell populations (5×10^4 and 10×10^5 cells/well, respectively). The detailed procedure and the method for removal of T_G cells have already been described.²⁷ The results are expressed as the absolute number of viable plasma cells per well.

Analysis of data. All data are presented as means $\pm$ SEM; comparisons between values were carried out by using either the Cockran-Cox test or Spearman's rank correlation test.

RESULTS

As shown in Fig 1 the mean value of sIL-2R in the serum of patients with B-CLL taken as a group was statistically higher than that obtained in age-matched controls (1,781 U/mL $\pm$ 231 v 276 U/mL $\pm$ 26; $P < .001$). When analyzed independently, the sIL-2R levels fall within the normal range in only three cases (5.5%). No statistically significant difference was observed between values detected in treated and untreated patients. Although patients with the lowest levels of sIL-2R were observed in stage 0, a direct correlation between the different stages of the disease and the sIL-2R values was not always found (Fig 2). The only significant difference was observed between stage 0 and stage IV values (932 U/mL $\pm$ 170 v 3,103 U/mL $\pm$ 1,021, respectively; $P < .05$).

The levels of sIL-2R in our patients were also compared in all cases with the number of WBC, in 47 cases with the T cell subset distribution, and in 49 cases with the serum immunoglobulin levels (Table 1). Although no statistically significant differences were observed between each subgroup because the sIL-2R levels were almost always higher than in normal controls, a general trend pointing to overall lower values in patients with less-invasive disease was observed. This was further confirmed by the rank correlation test (Table 1), determined in all patients taken as a group, that showed that the serum levels of sIL-2R correlated significantly with WBC counts and the levels of serum immunoglobulins ($r_s = .29$ and $.33$, respectively; $P < .05$); no significant correlation was demonstrated with the T cell subset distribution. When a correlation was made between sIL-2R levels and the T and B lymphocyte numbers, the statistical signifi-

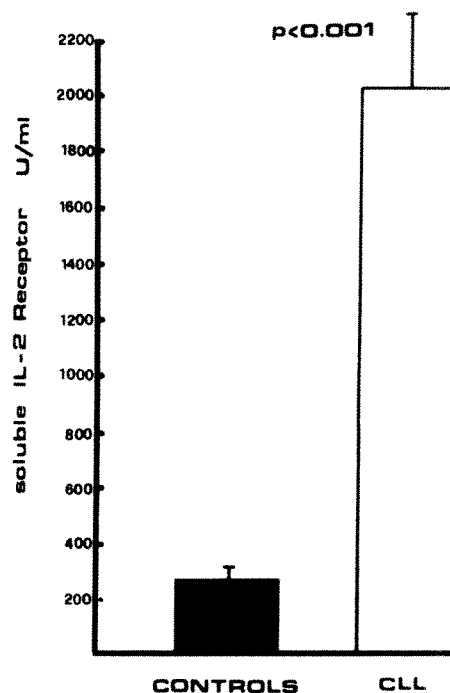


Fig 1. Serum levels of sIL-2R in B-CLL patients considered as a group and in controls ($P < .001$).

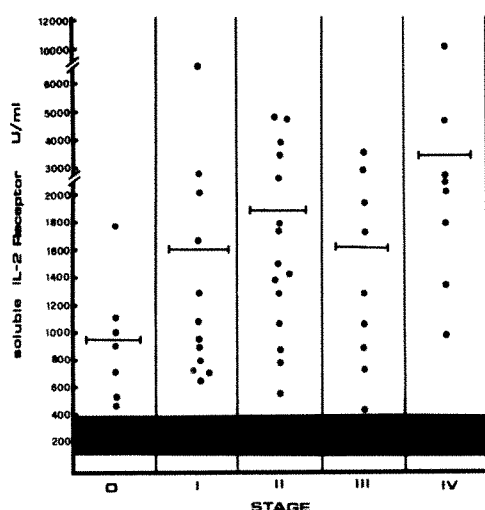


Fig 2. Serum levels of sIL-2R in B-CLL patients according to the different stages of the disease defined by Rai's system. The only significant difference was found between stages 0 and IV ($P < .05$). The shaded area indicates the control range.

cance did not differ from that observed comparing sIL-2R levels and the whole number of WBC.

As illustrated in Fig 3, the levels of sIL-2R were also correlated with in vitro functional T cell activities. The analysis of sIL-2R levels and functional in vitro studies were performed at the same time. Panel A illustrates the different patterns observed in patients tested for their proliferative response to PHA. Although in a few patients the enriched T cells displayed a normal in vitro blastogenesis, most of them showed impaired ^3H -thymidine incorporation. Although in the majority of cases with high levels of sIL-2R the response to PHA was very low, a strict correlation between the sIL-2R levels and this in vitro function was not always observed (Fig 3, panel A: $r_s = .34$; P is not significant). It is worth noting,

Table 1. sIL-2R Values in Different Groups of B-CLL Patients Subdivided According to Different Hematologic Parameters

Different Groups of B-CLL Patients	n	sIL-2R Values* (U/mL)	r_s †
Patients with WBC ($\times 10^9/\text{L}$)			
<25	14	1,157 $\pm$ 229	
25-49	20	1,937 $\pm$ 495	.29 ($P < .05$)
50-75	11	1,895 $\pm$ 382	($n = 54$)‡
>75	9	2,264 $\pm$ 603	
Patients with CD4/CD8 ratio			
>1.1	17	1,257 $\pm$ 217	
1.1-0.5	19	2,049 $\pm$ 491	.23 ($P = \text{NS}$)
<0.5	11	1,709 $\pm$ 354	($n = 47$)‡
Patients with serum Ig (mg/100 mL)			
>15	14	1,073 $\pm$ 161	
15-8	24	2,006 $\pm$ 324	.33 ($P < .05$)
<8	11	2,597 $\pm$ 796	($n = 49$)‡

*Mean $\pm$ SE. The mean values among different groups were not statistically significant.

†Correlation between sIL-2R and different hematologic parameters. Spearman's rank correlation test was used.

‡All patients taken as a group.

however, that the two patients with the lowest levels of sIL-2R showed a normal or near-normal response to PHA.

With regard to the ability of T cells from B-CLL to provide helper activity in a PWM-induced B cell differentiation assay (Fig 3, panel B), most cases displayed an impaired function. Again, the majority of patients with reduced helper function showed high levels of sIL-2R ($r_s = .43$; P , not significant), whereas the two patients with lowest values of sIL-2R displayed a normal or near-normal differentiation capacity.

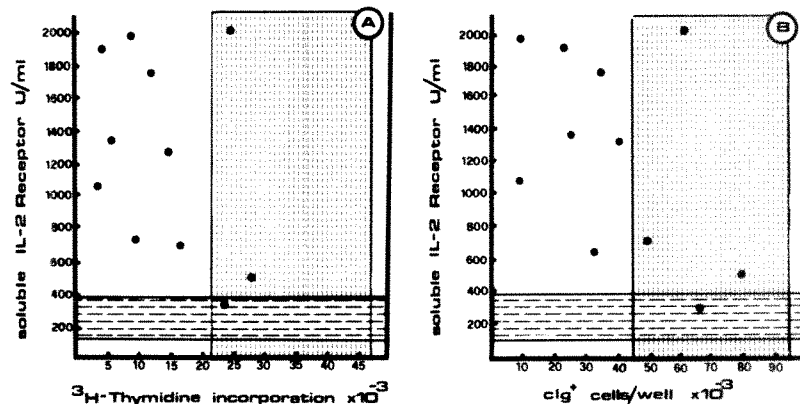
DISCUSSION

In this study we documented that patients with B-CLL display serum levels of sIL-2R that are significantly higher than in normal, age-matched controls. This finding confirms and extends a recent observation by Greene et al.²⁵ The presence in the serum of these patients of high quantities of sIL-2R may, in turn, play a role in the establishment of a number of functional abnormalities that have been described within the residual T cell population in B-CLL. Although the increase in sIL-2R was found in all stages of the disease, the lowest levels of sIL-2R were nonetheless observed in patients in stage 0, with a WBC count lower than $25 \times 10^9/\text{L}$, with a CD4/CD8 ratio greater than 1.1, and with serum immunoglobulin values higher than 15 g/100 mL. Furthermore, when the sIL-2R was correlated with the functional status of B-CLL T lymphocytes, it was found that patients with the lowest levels showed the best response to PHA and helper capacity.

Although the exact function of sIL-2R remains to be clarified, the ability of this molecule to bind to IL-2³⁰ suggests a potential role in the regulation of IL-2-dependent cell functions. The increased levels of sIL-2R found in B-CLL, associated with the evidence that B-CLL cells are capable of expressing IL-2R and absorbing variable amounts of IL-2,^{7,15} may play a role towards removing the available IL-2. This would represent a reasonable explanation for some of the immunologic functional abnormalities reported in this disease, which are basically mediated by IL-2. In line with our suggestion, it has been demonstrated that the addition of exogenous IL-2 is capable of partially correcting the in vitro T cell proliferative response to mitogens,¹⁶ of inducing a cytotoxic activity,³¹ and of improving T cell colony growth.³²

A major problem that remains is the definition of the type of IL-2 receptors in B-CLL. It has been demonstrated recently that different affinity forms of the IL-2 receptor exist, notably high- and low-affinity receptors.³³ Only the high-affinity receptors seem to mediate the growth-promoting response to IL-2, whereas the function of low-affinity receptors remains undefined.^{29,33-35} We presently do not know the precise form and the actual function of the sIL-2R present in B-CLL sera. However, preliminary studies performed in our laboratory point to the immunoregulatory role of sIL-2R in these patients. In fact, B-CLL sera were able to inhibit the proliferative in vitro response of normal lymphocytes to PHA, the highest inhibitory capability being provided by sera with the highest levels of sIL-2R. The inhibi-

Fig 3. Correlations between serum levels of sIL-2R and in vitro functional activities. Panel A correlates with the proliferative response to PHA. Panel B correlates with the ability of T cells from B-CLL patients to provide helper activity in a PWM-induced B cell differentiation assay. The shaded areas indicate different control ranges.



tory capacity was abrogated by the previous incubation of B-CLL sera with anti-Tac MoAb (data not shown).

A possible explanation for the lack of a strict correlation between serum levels of sIL-2R and relevant clinicoimmunologic features (Fig 2 and Table 1) is that the suggested defective availability of IL-2 may interfere with a large number of immunologic functions. As a result, the complex balance of immunoregulatory circuits makes it difficult to correlate strictly the serum levels of sIL-2R with individual immunologic parameters. The defective availability of IL-2

due to the presence of increased levels of sIL-2R may affect both the residual T and B cell populations in B-CLL patients, ultimately leading to defective immunoregulation, which may account for the hypogammaglobulinemia, susceptibility to infections, and incidence of second neoplasia often observed in this disease.^{4,36-39}

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The Effects of Purified Recombinant Murine Interleukin-3 and/or Purified Natural Murine CSF-1 In Vivo on the Proliferation of Murine High- and Low-Proliferative Potential Colony-Forming Cells: Demonstration of In Vivo Synergism

By Douglas E. Williams, Gao Hangoc, Scott Cooper, H. Scott Boswell, Richard K. Shadduck, Steven Gillis, Abdul Waheed, David Urdal, and Hal E. Broxmeyer

Purified natural murine L cell (macrophage) colony-stimulating factor (nCSF-1) and purified recombinant murine interleukin-3 (rIL-3) were administered to normal or lactoferrin-pretreated mice 20 to 24 hours before sacrifice. rIL-3 and nCSF-1 administered separately increased the percentage of macrophage high-proliferative potential colony-forming cells (HPP-CFC) and low-proliferative potential colony-forming cells (LPP-CFC) in active cell cycle. Endotoxin was not detected in the samples of nCSF-1 or rIL-3 with the *Limulus* lysate test, and the in vitro and in vivo hematopoietic stimulatory effects of both molecules were

abolished or markedly reduced by 30 minutes' treatment at 100°C, which demonstrates that the effects noted in vivo were not due to endotoxin. Combinations of low concentrations of rIL-3 and nCSF-1, which by themselves were inactive, increased the percentage of HPP-CFC and LPP-CFC in active cell cycle in a synergistic fashion. No significant change in the number of HPP-CFC or LPP-CFC per femur or femoral nucleated cellularity was observed. Thus, rIL-3 and nCSF-1 can synergize to effect the proliferation of the same cell populations in vivo.

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MURINE MACROPHAGE PROGENITOR cells with a high proliferative potential (HPP-CFC) can be detected in normal bone marrow and 5-fluorouracil-treated bone marrow.¹ HPP-CFC represent a developmentally early cell in the hematopoietic stem and progenitor cell hierarchy^{1,2} and form large (≥ 0.5 mm in diameter) colonies of macrophages in vitro. Colony formation is dependent upon a source of macrophage colony-stimulating factor (CSF-1) and a synergistic factor (SF).¹ SF has been detected in media conditioned by the myelomonocytic leukemia cell line WEHI-3B, and interleukin-3 (IL-3) purified from WEHI-3B³ or recombinant murine IL-3 can act as an SF in the presence of CSF-1.⁴ These data along with the biochemical similarities of SF and IL-3⁵ suggest that they are the same molecule.

Purified recombinant murine IL-3 (rIL-3) and purified natural murine L cell CSF-1 (nCSF-1) have recently been shown to influence progenitor cell (CFU-GM, BFU-E, CFU-GEMM, CFU-S) cycling and progenitor cell number in mice receiving either a single intravenous (IV) injection,⁶⁻⁸ multiple injections,⁹ or continuous infusion¹⁰ of one of these factors. These studies have all concentrated on the effects of a single type of CSF and have not examined the actions of these molecules on the HPP-CFC. In the present studies, we demonstrate that a single injection of rIL-3 or nCSF-1 increases the cycling status of HPP-CFC and low-proliferative potential colony-forming cells (LPP-CFC, a subpopulation of CFU-GM) when injected into normal or lactoferrin (LF)-pretreated mice. In addition, low concentrations of combinations of rIL-3 and nCSF-1 that were not active when administered separately acted synergistically in vivo in LF-treated mice to increase the percentage of HPP-CFC and LPP-CFC in active cell cycle.

MATERIALS AND METHODS

Animals. Female (C57BL/6J $\times$ DBA/2) F₁ (BDF₁) mice 6 to 8 weeks of age were purchased from Cumberland View Farms, Clinton, TN.

CSFs. nCSF-1¹¹ and rIL-3,¹² with specific activities of 2.3×10^7 U/mg and 1×10^9 U/mg, respectively, were devoid of detectable bacterial endotoxins (ET) as measured by the *Limulus* amebocyte lysate test (<0.5 ng/mL, Sigma Chemical Co, St Louis).

Experimental procedure. Animals received the appropriate concentration of CSF or pyrogen-free saline 20 to 24 hours before sacrifice. Maximal effects of the CSFs occur at this time when given as a single injection.^{6,7} Both femurs were removed, and cells were carefully flushed from the femoral cavity with ice-cold McCoy's 5A medium (GIBCO, Grand Island, NY). Single-cell suspensions were prepared by passage through a 23-gauge needle. Cells from the two femurs of individual mice were counted with a Coulter F_n particle counter and the number of nucleated cells per femur determined for each mouse.

Determination of the percentage of cells in S phase. The cycling status of progenitor cells was determined by the high-specific activity, tritiated-thymidine (³HTdR) kill technique as described.⁶ Briefly, 2×10^6 cells in 0.5 mL of McCoy's 5A medium supplemented with 10% heat-inactivated (56°C, 30 minutes) fetal bovine serum (HIFBS, Hyclone, Logan, UT) were incubated at 37°C for 20 minutes in the presence and absence of 50 μ Ci of ³HTdR (20 Ci/mmol, New England Nuclear, Boston). Unlabeled thymidine (2.5 mg) was added, the cells were washed twice and plated in a colony-forming assay. The percent reduction in colony number in cultures of cells incubated with ³HTdR, compared with unlabeled thymidine only, was taken as a measure of the percentage of cells in S phase.

Murine HPP-CFC culture system. HPP-CFC were grown in a

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double-layer agar culture system as previously described.¹ Optimal concentrations of prescreened L929 conditioned medium as a source of CSF-1 and WEHI-3B conditioned medium as a source of SF (IL-3) were included in the bottom layer. Cells (25,000 per plate) were incorporated into the agar overlayer and incubated in a humidified atmosphere of 5% CO₂ in air for 14 days. HPP-CFC were scored at 40× with a dissecting microscope and were defined as tightly packed aggregates of >0.5 mm overall diameter. Loosely arranged aggregates, or those ≤0.5 mm but >50 cells were scored as low-proliferative potential colony-forming cells (LPP-CFC). In some studies, the macrophage composition of HPP-CFC colonies was confirmed by staining with nonspecific esterase.

RESULTS

Single intravenous injections of 2×10^4 units of nCSF-1 or 2×10^3 units of rIL-3 were administered to untreated BDF₁ mice. These concentrations produce maximal effects on CFU-GM, BFU-E, and CFU-GEMM.⁶ HPP-CFC went from a noncycling state in saline-treated control animals ($3\% \pm 3\%$ cycling) to $40\% \pm 3\%$ ($P < .001$) in cycle after rIL-3 treatment and $33\% \pm 6\%$ ($P < .001$) in cycle after nCSF-1 treatment. LPP-CFC (CFU-GM) were $45\% \pm 1\%$ cycling in saline-treated animals and increased to $50\% \pm 2\%$ ($P < .05$) after rIL-3 and $53\% \pm 1\%$ ($P < .05$) after nCSF-1 treatments.

LF acts in vivo to decrease the percentage of LPP-CFC (CFU-GM) in active cell cycle by increasing the sensitivity of these cells to exogenous CSF.⁶ LF does not effect the HPP-CFC cycling status, since these cells are noncycling cells in untreated mice ($3\% \pm 3\%$ cycling minus LF, $6\% \pm 6\%$ cycling plus LF), and does not alter the number of HPP-CFC per femur ($13.3 \pm 0.4 \times 10^3$ minus LF, $12.9 \pm 2.0 \times 10^3$ plus LF). Therefore, to compare the possible synergistic proliferative effects of nCSF-1 and rIL-3 in vivo on both HPP-CFC and LPP-CFC, mice were pretreated with LF. Injection of as little as 1,000 units nCSF-1 or 100 units rIL-3 stimulated HPP-CFC and LPP-CFC into an active cycling state (Table 1). When nCSF-1 and rIL-3 were administered to mice simultaneously, the concentration of nCSF-1 and rIL-3 necessary to significantly increase the percentage of HPP-CFC or LPP-CFC in cycle was reduced by 20-fold and

tenfold, respectively (Table 1). The combination of nCSF-1 and rIL-3 at concentrations that were not themselves active resulted in a significant stimulation of the percentage of HPP-CFC and LPP-CFC in active cell cycle. No significant effects on the number of progenitors per femur or total femoral nucleated cellularity were observed when using these concentrations of nCSF-1 or rIL-3 (Table 1).

As measured by the *Limulus* lysate test, nCSF-1 and rIL-3 samples contained no detectable ET (<0.5 ng/mL or <0.1 ng/injection). However, the same concentrations (ng/mL) of different ET preparations assay differently with the *Limulus* test,¹³ and polynucleotides or proteins can interfere with this ET assay system.¹⁴ As an additional ET control, CSF samples for injection were boiled for 30 minutes. This treatment will not destroy ET¹⁵ but ablated the in vivo effect on HPP-CFC cycling exerted by 2×10^4 units nCSF-1 ($44\% \pm 8\%$ cycling minus heating, $0\% \pm 3\%$ cycling plus heating) in previously untreated mice. Bone marrow colony formation stimulated by 100 units nCSF-1¹¹ was reduced by 92% (124 ± 3 to 11 ± 2 colonies), and rIL-3-stimulated FDC-P1 proliferation¹² was reduced from a plateau level of 1.65×10^5 cpm to background levels after heat treatment.

DISCUSSION

The present studies were performed to investigate the in vivo effects of low concentrations of nCSF-1 and rIL-3 on primitive macrophage progenitor cells (HPP-CFC) and more mature LPP-CFC. Both nCSF-1 and rIL-3 when given alone increased the percentage of HPP-CFC and LPP-CFC in active cell cycle when administered to mice. These in vivo effects could be abolished by boiling the CSFs for 30 minutes, thereby demonstrating that the observed in vivo effects were not the result of bacterial ET since boiling is insufficient to inactivate the in vivo actions of ET.¹⁵ In vitro biologic activity of nCSF-1 or rIL-3 was also partially or completely lost upon heat treatment, which paralleled the effects observed in vivo. These heat treatment studies suggest that the observed effects of rIL-3 and nCSF-1 in vivo are not a nonspecific stimulating effect of proteins in general.

The synergistic influence of CSF-1 and IL-3 on the in vitro

Table 1. The Effects of nCSF-1 and rIL-3 Administered Separately or Together on Murine HPP-CFC and LPP-CFC

Experimental Group	Nucleated Cells ($\times 10^6$ per Femur)	HPP-CFC ($\times 10^3$ per Femur)	Percent HPP-CFC in Cycle	LPP-CFC ($\times 10^3$ per Femur)	Percent LPP-CFC in Cycle
Saline (control)	17.6 ± 2.8	12.9 ± 2.0	6 ± 6	41.1 ± 6.5	5 ± 7
CSF-1, 2,000 U	17.3 ± 1.7	12.8 ± 1.2	$43 \pm 8^*$	40.7 ± 4.0	$47 \pm 9^*$
CSF-1, 1,000 U	18.2 ± 2.0	13.7 ± 1.5	$37 \pm 10^*$	40.4 ± 4.4	$36 \pm 9^*$
CSF-1, 500 U	18.3 ± 2.4	11.1 ± 1.4	4 ± 7	42.3 ± 2.0	4 ± 4
IL-3, 200 U	17.3 ± 1.3	11.8 ± 0.9	$38 \pm 4^*$	44.3 ± 4.2	$35 \pm 5^*$
IL-3, 100 U	16.1 ± 1.4	11.8 ± 3.2	$22 \pm 8^\dagger$	40.1 ± 3.5	$31 \pm 6^*$
IL-3, 50 U	17.1 ± 2.6	11.0 ± 1.7	7 ± 6	44.8 ± 6.8	3 ± 3
500 U CSF-1 + 50 U IL-3	17.4 ± 2.0	11.2 ± 1.3	$37 \pm 6^*$	41.3 ± 4.7	$41 \pm 6^*$
100 U CSF-1 + 25 U IL-3	16.3 ± 1.3	11.2 ± 0.8	$30 \pm 13^*$	37.5 ± 3.0	$35 \pm 5^*$
50 U CSF-1 + 10 U IL-3	17.5 ± 4.3	12.7 ± 2.4	$23 \pm 12^\dagger$	42.3 ± 1.0	$22 \pm 12^\dagger$
10 U CSF-1 + 1 U IL-3	16.3 ± 1.6	10.5 ± 1.0	4 ± 5	38.4 ± 3.8	2 ± 5

Mice received pyrogen-free saline or test material IV 20 to 24 hours before death. Three hours earlier, 50 μ g of ET-depleted, iron-saturated LF was administered IV. In mice given nCSF-1 plus rIL-3, separate injections were performed for each factor, one immediately after the other. Each mouse was assayed individually, and data are reported as means $\pm$ SD. There was a total of six to ten mice per experimental group in two experiments.

*Significantly different from control, $P < .001$.

†Significantly different from control, $P < .05$.

proliferation of murine HPP-CFC is well documented.¹⁻⁵ Recent studies have also demonstrated that these two factors can have a greater than additive or synergistic effect on the in vitro proliferative potential of CFU-GM.^{16,17} The present studies demonstrated that nCSF-1 and rIL-3 could synergize in vivo to increase the percentage of HPP-CFC and LPP-CFC (CFU-GM) in active cell cycle. Concentrations of either CSF that had no activity when administered alone exhibited strong stimulatory effects on progenitor cell cycling if given together, in accordance with the definition of a synergistic interaction.¹⁸ The in vivo stimulatory effects of

rIL-3 and nCSF-1 alone or in combination may result from direct effects on the progenitor cells or effects on accessory cells. Synergistic responses to multiple CSF species are probably of relevance physiologically and may be important clinically.

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Recombinant Human Granulocyte Colony-Stimulating Factor Repairs the Abnormalities of Neutrophils in Patients With Myelodysplastic Syndromes and Chronic Myelogenous Leukemia

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We examined the in vitro effect of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on neutrophil anomalies in 20 patients with myelodysplastic syndromes (MDS) and eight patients with chronic myelogenous leukemia (CML). Neutrophil alkaline phosphatase (NAP) activity was determined in nine MDS patients and eight CML patients by a scoring method. NAP scores were decreased in six of the nine patients with MDS and in all of the patients with CML. In all patients with these diseases, NAP scores increased by incubating the blood with rhG-CSF. An increase in NAP scores by rhG-CSF was observed even at a concentration of 1 U/mL in patients with MDS but was observed only at higher concentrations (1,000 to 10,000 U/mL) in patients with CML. Significant increases in NAP scores occurred at 12 hours' incubation in patients with MDS, whereas the increase was more gradual in patients with CML. This time course difference was thought to be due mainly to the difference in cell populations of circulating myeloid cells between MDS patients and CML patients. Induction of NAP activity

by rhG-CSF in patients with both these diseases was suppressed by the addition of inhibitors of RNA or protein synthesis. Neutrophil superoxide anion (O_2^-) production induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was determined in the other 11 patients with MDS. This neutrophil function was decreased in seven of the 11 patients with MDS, normal in two patients, and increased in two patients. Preincubation with rhG-CSF caused a significant increase in fMLP-induced O_2^- production in nine of the 11 patients with MDS. rhG-CSF enhanced this neutrophil function in a time- and dose-dependent manner, and maximal stimulation was observed at 2,000 to 4,000 U/mL of rhG-CSF and at five to ten minutes' incubation. The present results show that rhG-CSF is able to repair at least in part the neutrophil anomalies in these patients, and our data, especially for patients with MDS, suggest the clinical usefulness of rhG-CSF for this preleukemic disorder.

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MYELODYSPLASTIC SYNDROMES (MDS) are preleukemic hematologic disorders that were proposed and classified by the French-American-British (FAB) cooperative group.¹ Various morphological,¹⁻³ cytochemical,^{2,6} and functional^{5,7-9} abnormalities of neutrophils have been observed in patients with MDS. Among them, neutrophil alkaline phosphatase (NAP) activity, which is decreased in patients with chronic myelogenous leukemia (CML) in chronic phase,^{10,11} is also found to be decreased frequently in patients with MDS.⁶

Recently, we and other laboratories have succeeded in cloning and expressing cDNA for human granulocyte colony-stimulating factor (G-CSF) in *Escherichia coli*, and large-scale production of recombinant human G-CSF (rhG-CSF) has been established.^{12,13} It is generally accepted that G-CSF is one member of a family of hemopoietic growth factors that are required for the proliferation and differentia-

tion of granulocyte and macrophage precursor cells.¹⁴ But Nicola et al reported that peripheral blood neutrophils also have specific receptors for G-CSF,¹⁵ and their observation suggests the possibility that human G-CSF plays some roles in circulating mature neutrophils as well as their progenitors. Therefore, it is important to determine the effects of rhG-CSF on circulating neutrophils. Moreover, in considering clinical application of human G-CSF to hematologic disorders, it is also important to investigate the effects of this factor on abnormal neutrophils.

To investigate whether rhG-CSF can repair neutrophil anomalies, we examined the in vitro effect of rhG-CSF on NAP activity in patients with MDS and CML and report here its ability to enhance decreased NAP activity in these patients. Furthermore, in this paper we also report that superoxide anion (O_2^-) production by neutrophils is frequently decreased in patients with MDS and that rhG-CSF is a potent stimulator of this neutrophil function.

MATERIALS AND METHODS

Preparation and purification of rhG-CSF. Human G-CSF was purified to homogeneity from a protein-free culture line, Blanche-1,¹⁶ and the amino-terminal sequence of human G-CSF was determined by a gas-phase sequencer (unpublished results, September 1985). By using poly(A)⁺ RNA prepared from human macrophages, a cDNA library was constructed by the method of Okayama and Berg.¹⁷ The resulting cDNA library was screened by the synthetic oligonucleotide probes, which were proposed by the amino-terminal sequence. The amino acid sequence deduced from the cloned G-CSF cDNA was identical to that reported by Souza et al.¹³ The G-CSF cDNA was expressed in *E. coli* K12 MM294 by construction of an expression plasmid for G-CSF using pGEL-1 containing the *trp* promoter,¹⁸ and the mature protein was purified according to the method of Marston et al.¹⁹ The purified rhG-CSF was found to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and have an apparent molecular weight of

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17,400.²⁰ Activity of rhG-CSF was defined according to the method of Nicola et al.,²¹ ie, the rhG-CSF concentration required for half-maximal stimulation of colony formation by 75,000 BDF₁ mouse (Charles River, Japan) bone marrow cells in semisolid agar culture was defined as 50 units. The purified rhG-CSF had a specific activity of 1×10^8 U/mg protein and contained less than 2.0 ng endotoxin/mg protein.²⁰

Patients. Nine patients with MDS and eight patients with CML were studied in the NAP experiment, and the other 11 patients with MDS were studied in the neutrophil O_2^- production experiment. The criteria for diagnosis of MDS were based on the report of the FAB group.¹ In all cases, hematologic studies were done on both bone marrow films and peripheral blood smears stained by the Wright-Giemsa method, and chromosomal analysis was performed in all patients with CML. For the control studies, 12 (NAP study) and 11 (O_2^- study) normal volunteers were compared; informed consent was obtained from all volunteers.

Preparation of cells. In the NAP experiment, mononuclear leukocytes (MNL) and polymorphonuclear leukocytes (PMNL) were simultaneously isolated from heparinized venous blood of CML patients by using Mono-Poly Resolving Medium (M-PRM; Flow Laboratories, North Ryde, Australia) by the method reported by Ferrante and Thong.²² Blood was layered carefully onto M-PRM placed in plastic tubes and then centrifuged at 300 g for 30 minutes at room temperature. The MNL fraction at the plasma-M-PRM interface and the PMNL fraction below the interface were separately transferred to individual tubes and washed once or twice with RPMI 1640 medium (GIBCO, Grand Island, NY). More than 95% of the leukocytes in the PMNL fraction were mature neutrophils (most of them were segmented neutrophils), as judged by Wright-Giemsa staining. The MNL fraction mainly contained myelocytes, metamyelocytes, basophils, monocytes, and lymphocytes. In the O_2^- production experiment, granulocytes were prepared as described.²³ In brief, the heparinized venous blood from MDS patients and healthy adult donors was allowed to sediment at room temperature for 40 to 50 minutes after mixing with a three-fourths volume of 1% dextran (Nakarai Chemical Co, Kyoto, Japan) in isotonic saline. The pure granulocyte fractions were obtained from leukocyte-rich supernates by the Ficoll-Conray method (Ficoll, Pharmacia Fine Chemicals, Piscataway, NJ; and Conray, Mallinckrodt Inc, St Louis), and contaminated erythrocytes were removed by hypotonic lysis. Granulocyte fractions were suspended in Hanks' balanced salt solution (HBSS; Nissui Seiyaku Co, Tokyo) and contained >98% granulocytes (>95% neutrophils).

Incubation in the NAP experiment. Whole blood from the patients and normal subjects was incubated in plastic tubes (2051; Falcon Labware, Oxnard, CA) for up to 48 hours in a fully humidified atmosphere of 5% CO_2 in air at 37°C with or without rhG-CSF. Fractionated leukocytes from venous blood of CML patients were incubated at cell densities of 1 to 2×10^6 /mL in RPMI 1640 medium supplemented with 10% fetal bovine serum in six-well tissue culture plates (3046; Falcon) under the same conditions. In some experiments, chemicals known to inhibit DNA, RNA, and protein synthesis were added to the cultures. These include hydroxyurea (Sigma Chemical Co, St Louis), actinomycin D (Calbiochem-Behring, La Jolla, CA), and cycloheximide (Sigma).

Determination of NAP activity. NAP-positive neutrophils were determined by the method reported by Tomonaga et al.²⁴ In brief, blood films or smears of cultured leukocytes were dried in air and fixed with 10% cold formalin in methanol and then incubated with a solution containing naphthol AS-MX phosphate (Muto Pure Chemicals, Tokyo) at 37°C for two hours. After incubation, blood films or leukocyte smears were stained with 1% aqueous safranin O for two minutes. Neutrophils showed alkaline phosphatase as a blue deposit. The degree of activity in each cell was rated from O to V on the basis

Table 1. Hematologic Data of the Patients With MDS

Patient	Diagnosis (FAB)	WBC (/μL)	Differential Count of WBC (%)				
			Band	Seg	Eo	Mo	Ly
1	RA	6,500	2	52	0	10	36
2	RA	2,000	3	41	2	2	52
3	RA	4,700	3	37	2	5	53
4	RA	11,900	5	30	45	4	16
5	RARS	2,800	4	46	0	2	48
6	RAEB	1,500	0	19	16	3	62
7	RAEB	2,000	1	46	0	4	49
8	RAEBinT	1,200	4	27	0	1	68
9	RAEBinT	1,700	13	54	0	2	31

Abbreviations: RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RAEB, refractory anemia with excess of blasts; RAE-BinT, refractory anemia with excess of blasts in transformation; Band, band-form neutrophil; Seg, segmented neutrophil; Eo, eosinophil; Mo, monocyte; Ly, lymphocyte.

of the number of the blue precipitated granules in the cytoplasm: O, no granules; I, up to five granules; II, easily counted number (up to approximately 30 granules); III, unevenly distributed; IV, almost evenly distributed; V, uniformly densely distributed. The sum of the rating of 100 cells was considered the NAP score for a particular smear.

Determination of O_2^- production. O_2^- was assayed spectrophotometrically by the reduction of cytochrome c (type III; Sigma), and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double-wavelength spectrophotometer; Hitachi Ltd, Tokyo) equipped with a thermostatted cuvette holder as described.²³ The cell suspension in HBSS was added to a 1-mL cuvette containing 110 μmol/L ferricytochrome c to obtain a final volume of 0.995 to 0.985 mL and a final cell concentration of 5×10^5 /mL. Stimulating agents (5 to 15 μL) were added to the reaction mixture in cuvettes to obtain the desired concentrations of the agents while the time course of cytochrome c reduction (the absorbance change at 550 to 540 nm) was followed on the recorder. We used *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma) as a stimulant, and its final concentration was 1 μmol/L in this study. Cytochrome c

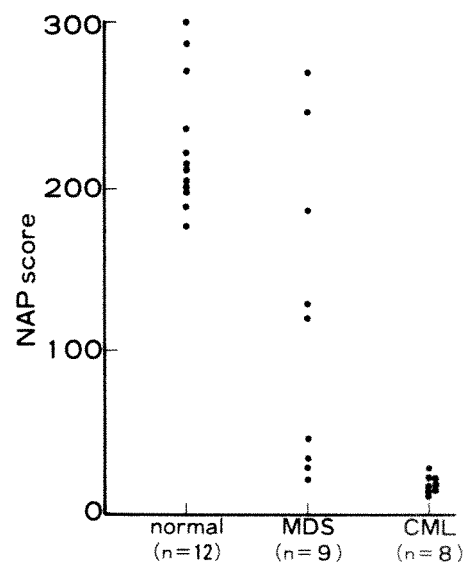


Fig 1. NAP scores of cells from 12 normal subjects, nine patients with MDS, and eight patients with CML before incubation.

reduction by neutrophils was completely abolished by superoxide dismutase (Sigma) and suggested to be specific for O_2^- . The production of O_2^- was calculated from cytochrome c reduced for five minutes after the addition of fMLP using an extinction coefficient of $21.1 \text{ mol/L}^{-1}/\text{cm}^{-1}$.²⁵

RESULTS

Effect of rhG-CSF on NAP activity in normal subjects and in patients with MDS and CML. All patients with MDS were diagnosed and subclassified according to FAB criteria.¹ As shown in Table 1, immature myeloid cells were not observed in the peripheral blood of the patients with MDS. All patients with CML had the Philadelphia (Ph¹) chromosome and showed typical hematologic features of the chronic phase. No evidence of infection was observed in any patient when this study was performed.

Figure 1 shows the NAP scores of 12 normal subjects, 9 patients with MDS, and 8 patients with CML before the incubation. The mean NAP score of the 12 normal subjects was 225 ± 38 (mean $\pm$ SD). NAP scores were markedly decreased in four of the nine patients with MDS and were slightly decreased in two of them. The other three patients showed no significant decrease in NAP scores. In all patients with CML, NAP scores were markedly decreased (19 ± 5).

To test whether rhG-CSF stimulates NAP activity of the patients with MDS and CML, whole blood was incubated for 24 hours with or without rhG-CSF (Fig 2). NAP scores of all patients with these diseases increased after incubation with rhG-CSF, whereas rhG-CSF was not able to increase the NAP scores of normal subjects. NAP scores of normal subjects decreased during incubation without rhG-CSF except in one case, and rhG-CSF appears to partly prevent this decrease, but this effect was not significant.

As shown in Fig 3A, rhG-CSF enhanced the NAP activity of the patients with MDS in a dose-dependent manner, and significant increase in NAP scores caused by rhG-CSF

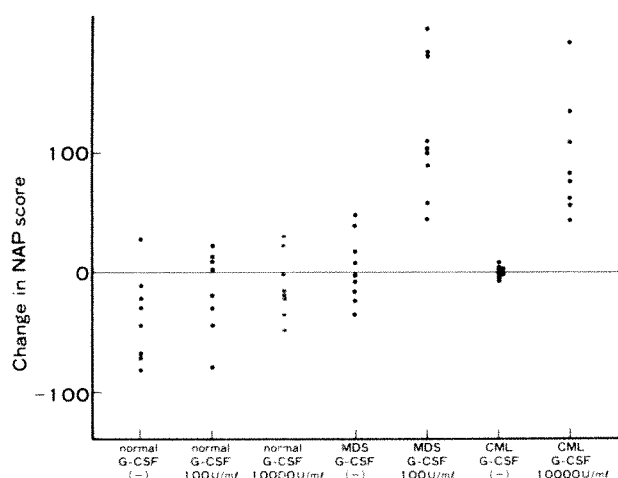


Fig 2. Changes in NAP scores in eight normal subjects, nine patients with MDS, and eight patients with CML during 24 hours' incubation with or without rhG-CSF. The positive figures designate NAP score increases and negative ones, decreases.

occurred at a concentration of 1 U/mL (0.01 ng/mL). In patients with CML, however, an increase in NAP scores was observed at more than 1,000 U/mL (10 ng/mL).

To exclude the influences of immature myeloid cells, the effect of rhG-CSF on mature neutrophils from six patients with CML was examined (Fig 3B). Although a slight to moderate increase in NAP scores was already observed at a concentration of 100 U/mL (1 ng/mL), dose-response curves were similar to those obtained when using whole blood of CML patients and apparently different from those observed in MDS patients.

NAP scores in patients with MDS began to increase at 12 hours' incubation, and maximal stimulation was observed at 24 hours, whereas in patients with CML, NAP scores increased significantly only at 24 hours' incubation and continued to increase thereafter (Fig 4).

Figure 5 shows the difference in time courses of an increase in NAP scores between MNL and PMNL from six patients with CML. The increase in PMNL NAP scores caused by rhG-CSF was almost maximal at 24 hours' incubation, whereas MNL NAP scores increased only slightly at 24 hours and a prominent increase was observed at 48 hours' incubation. Most of the NAP-positive MNL after incubation with rhG-CSF were myelocytes or metamyelocytes, and increases in MNL NAP scores were thought to reflect the increase in NAP activity of these immature myeloid cells.

To elucidate the mechanism of the induction of NAP activity by rhG-CSF, various inhibitors affecting different kinds of cell metabolism were added to the culture (Table 2). Induction of NAP activity by rhG-CSF in patients was inhibited by the addition of actinomycin D and cycloheximide, although delayed addition of actinomycin D failed to inhibit the increase in NAP activity. Hydroxyurea did not suppress the effect of rhG-CSF on NAP activity in these patients.

Effect of rhG-CSF on neutrophil O_2^- production in normal subjects and in patients with MDS. As shown in Table 3, the mean value of fMLP-induced O_2^- production by neutrophils from 11 normal subjects was 1.56 ± 0.25 (mean $\pm$ SD), and preincubation with rhG-CSF significantly stimulated this function of normal neutrophils. Neutrophils were preincubated with 2,000 U/mL (20 ng/mL) of rhG-CSF for five minutes because our previous results showed that an almost maximal priming effect of rhG-CSF was observed at this concentration and this incubation period.²⁶ In patients with MDS, neutrophil O_2^- production was significantly decreased in seven of the 11 patients and was significantly increased in two patients. The other two patients showed normal O_2^- production. After incubation with rhG-CSF, O_2^- production by neutrophils significantly increased in nine of the 11 patients with MDS. The other two patients showed no significant change in O_2^- production.

Figure 6 shows a representative time course of fMLP-induced O_2^- production by neutrophils in a patient. Preincubation with rhG-CSF apparently enhances reduction of cytochrome c as shown in this figure.

rhG-CSF enhanced O_2^- production by patients' neutro-

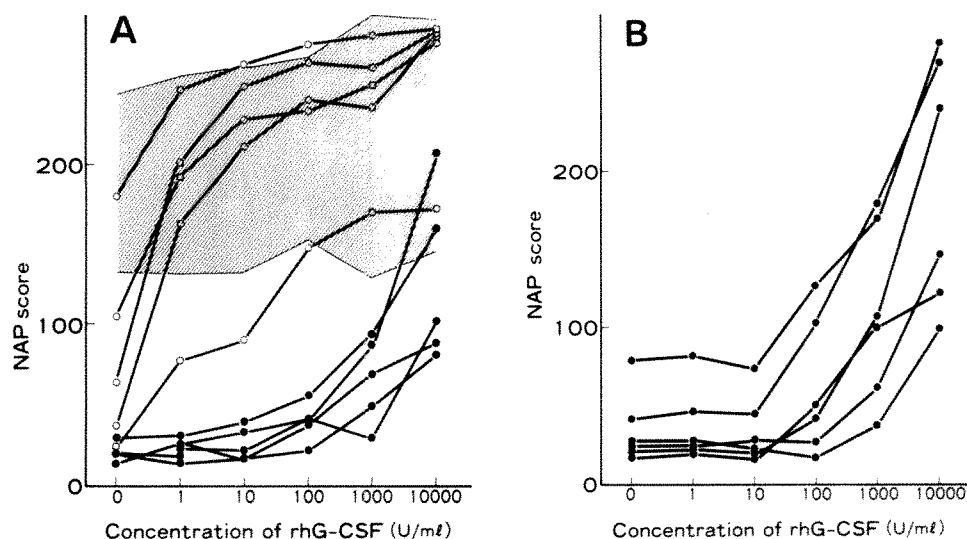


Fig 3. Dose-response curves of the effect of rhG-CSF on NAP scores in patients with MDS (○) and CML (●). (A) Whole blood from five patients with each disease was incubated for 24 hours with various concentrations of rhG-CSF. The oblique lined area designates the normal value (mean $\pm$ 2 SD of NAP scores after incubation in eight normal subjects). (B) Morphologically mature neutrophils in six patients with CML isolated as described in Materials and Methods were incubated for 24 hours with various concentrations of rhG-CSF.

phils in a dose-dependent manner, and maximal stimulation was observed at 2,000 to 4,000 U/mL (Fig 7). Slight increases in O_2^- production occurred at two minutes' incubation with rhG-CSF, and its enhancing effect was almost maximal at five to ten minutes (Fig 8).

DISCUSSION

Several investigators have reported that unknown extrinsic factors can increase NAP activity in patients with CML,²⁷⁻²⁹ and the possibility that CSF is one of these extrinsic factors has been discussed.^{30,31} Our in vitro data show that rhG-CSF can increase NAP activity in patients

with MDS and CML and this phenomenon, especially observed in patients with MDS, has not been reported previously.

The increase in NAP activity caused by rhG-CSF in the cells of these patients was almost completely inhibited by protein synthesis inhibitor when added at the beginning of incubation, and significant inhibition was also observed by the delayed addition of it. Interestingly, an inhibitor of RNA

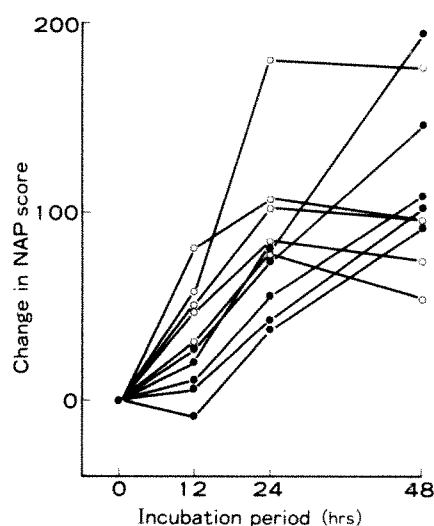


Fig 4. The time courses of increases in NAP scores for five patients with MDS (○) and five with CML (●). Whole blood from MDS patients was incubated with 100 U/mL rhG-CSF, and that of CML patients was incubated with 10,000 U/mL of it.

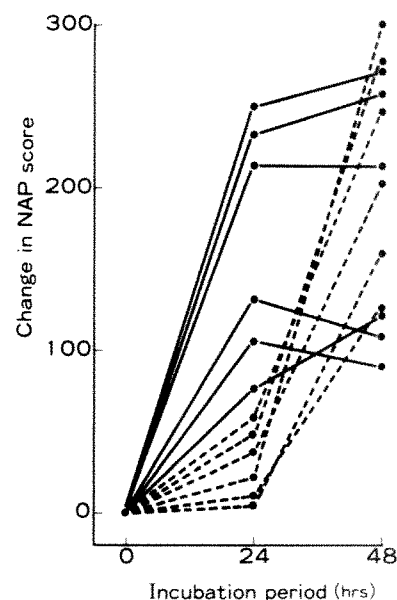


Fig 5. The time courses of increases in NAP scores by 10,000 U/mL rhG-CSF in six patients with CML. Solid lines show increases in NAP scores of mature neutrophils, and broken lines show those of immature myeloid cells from these patients. Mature neutrophils and immature myeloid cells were isolated from whole blood of the patients as described in Materials and Methods.

Table 2. Effects of Hydroxyurea, Actinomycin D, and Cycloheximide on the Induction of NAP Activity by rhG-CSF in Patients With MDS and CML

Agent Added	Time of Addition	Increase in NAP Score			
		MDS Patients		CML Patients	
Control (no agent)		180 (0.0)	202 (0.0)	186 (0.0)	75 (0.0)
Hydroxyurea (2 mmol/L)	0 h	158 (12.3)	182 (10.0)	180 (3.3)	78 (-4.0)
	12 h	168 (6.7)	178 (11.9)	191 (-2.7)	66 (12.0)
Actinomycin D (1 µg/mL)	0 h	-14 (107.8)	64 (68.3)	24 (87.1)	8 (89.3)
	12 h	144 (20.0)	193 (12.3)	209 (-12.3)	71 (5.3)
Cycloheximide (5 µg/mL)	0 h	-11 (106.1)	9 (95.9)	-1 (100.5)	7 (90.7)
	12 h	47 (74.0)	104 (48.5)	76 (59.2)	22 (70.7)

Blood was incubated with rhG-CSF at a concentration of 100 U/mL in patients with MDS and 10,000 U/mL in patients with CML with or without the agent that was added at the time indicated. NAP activity was determined after 24 hours' incubation. Percent inhibition was calculated by using the NAP score increase caused by rhG-CSF alone as a control and is shown in parentheses. This experiment was performed on two patients with MDS and two patients with CML.

synthesis that suppressed the effect of rhG-CSF when added at the start of culture could not inhibit the induction of NAP activity when added after 12 hours of incubation. These data indicate that de novo synthesis of RNA and protein was needed for the induction of NAP activity by rhG-CSF in

Table 3. Effect of rhG-CSF on fMLP-Induced O_2^- Production by Neutrophils in Patients With MDS

Patient	Diagnosis (FAB)	Neutrophil O_2^- Production	
		Control	G-CSF(+)
10	RA	0.41 ± 0.02*	0.94 ± 0.16†
11	RA	0.46 ± 0.01*	1.11 ± 0.12†
12	RA	0.70 ± 0.11*	1.41 ± 0.28‡
13	RA	0.94 ± 0.14*	2.20 ± 0.32†
14	RA	1.70 ± 0.47	2.87 ± 0.06‡
15	RA	1.75 ± 0.11	3.65 ± 0.75‡
16	RA	2.76 ± 0.51*	3.98 ± 0.19‡
17	RA	3.01 ± 0.25*	5.04 ± 0.31†
18	RAEB	0.15 ± 0.07*	0.20 ± 0.01
19	RAEB	0.87 ± 0.03*	1.20 ± 0.05†
20	RAEB	0.96 ± 0.10*	1.11 ± 0.11
Normal subjects (n = 11)		1.56 ± 0.25	2.91 ± 0.57†

Neutrophilic granulocytes were preincubated with rhG-CSF (2,000 U/mL) for five minutes at 37°C before fMLP (1 µmol/L) was added. O_2^- production is expressed as nmol cytochrome c reduced/5 min/5 × 10⁵ cells. Results from each experiment, done in triplicate, are shown, and each value represents the mean ± SD. The mean value (±SD) of neutrophil O_2^- production in 11 normal subjects is also shown. Significance was determined by Student's *t* test.

*Significantly decreased or increased compared with normal values obtained from healthy donors (*P* < .01).

†Significantly increased compared with the control value of the same patient (*P* < .01).

‡Significantly increased compared with the control value of the same patient (*P* < .05).

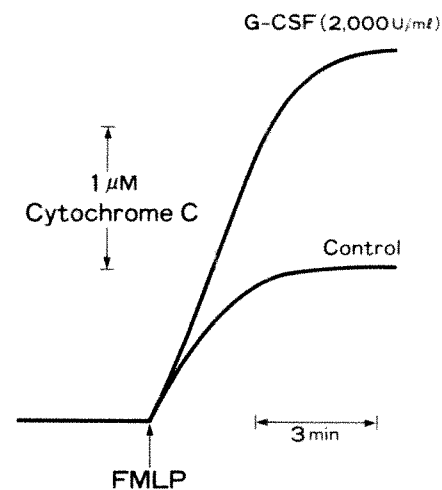


Fig 6. Time courses of neutrophil O_2^- production induced by fMLP with or without rhG-CSF pretreatment in a patient with MDS. Preincubation with rhG-CSF (2,000 U/mL) was performed for five minutes.

these patients and that RNA synthesis preceded protein synthesis. In contrast, DNA synthesis does not seem to occur during the induction of NAP activity. These results are consistent with the previous observation from normal human bone marrow neutrophils stimulated by retinoic acid³² and suggest that induction of NAP activity is brought out directly by the synthesis of this enzyme protein through common underlying mechanisms.

An increasing effect of rhG-CSF on NAP activity in patients with MDS was observed at a low concentration that could not support the colony formation and was also observed within 12 hours' incubation. Therefore, this effect of rhG-CSF seems to be very important, at least in patients with

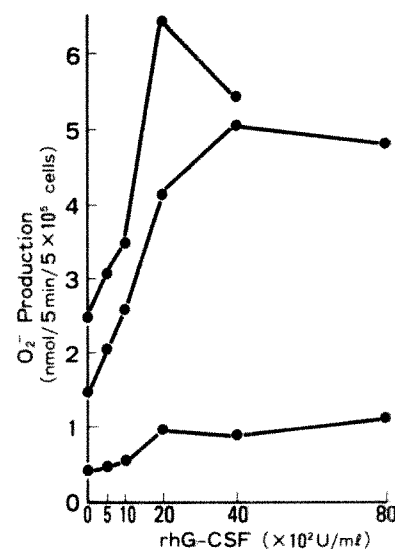


Fig 7. Effect of rhG-CSF on fMLP-induced O_2^- production in three patients with MDS. Neutrophils were preincubated with the indicated concentrations of rhG-CSF for five minutes at 37°C before fMLP was added. Each point represents the mean of duplicate experiments.

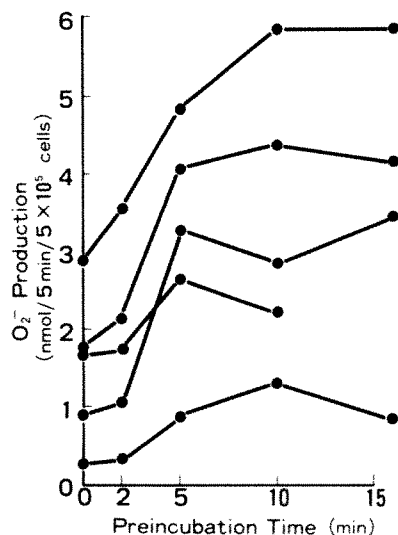


Fig 8. Effect of preincubation time with rhG-CSF on fMLP-induced O_2^- production in five patients with MDS. Neutrophils were preincubated with rhG-CSF (2,000 U/mL) for the indicated periods at 37°C before fMLP was added. Each point represents the mean of duplicate experiments.

MDS. Moreover, the circulating level of G-CSF in these patients is thought to be less than 1 U/mL (0.01 ng/mL) and insufficient to enhance the activity of this neutrophil enzyme since incubations were performed with whole blood from patients that contained its own G-CSF. On the contrary, an increase in NAP activity in patients with CML occurred only at higher concentrations of rhG-CSF. Its increase was minimal at 12 hours and was observed even at 24 to 48 hours' incubation. This difference in time courses seems to be due mainly to the difference in cell population of circulating myeloid cells in these two diseases; NAP scores of immature myeloid cells, which were observed only in patients with CML, increased prominently at 24 to 48 hours' incubation, and the time courses of the increase in NAP scores of mature neutrophils from CML patients resemble those of MDS patients. The difference in dose response is not attributable only to the difference in cell population since the lower concentrations of rhG-CSF could not induce NAP activity in patients with CML even when the study was performed with pure neutrophil fractions. This difference in response to G-CSF will have some relation to differences in the receptor-signal transduction system or other cell metabolism in neutrophils and may reflect the different mechanism of NAP deficiency in these two hematologic disorders.

NAP has been thought to be a marker of neutrophil maturity,^{33,34} and de novo synthesis of this enzyme protein seems to be necessary for its induction, based on the results of a previous report³² and our present study. Another previous observation was that amino acid incorporation of normal circulating leukocytes is minimal and much less than that of leukemic white cells from CML patients.³⁵ These results may lead us to hypothesize that normal circulating neutrophils with normal NAP activity are fully mature and have little ability to synthesize this enzyme protein further and that circulating leukemic neutrophils with low NAP activity are

immature and have the potential to synthesize this enzyme. Our in vitro data obtained from neutrophils of normal subjects and morphologically immature neutrophils of CML patients support this hypothesis. However, morphologically mature neutrophils in patients with MDS and CML also showed low NAP scores and were induced to increase their activity by rhG-CSF. These abnormal neutrophils apparently have a discrepancy in maturity between their morphology and NAP activity. Recently, Berkow and Dodson reported that morphologically mature neutrophils from the bone marrow of a healthy donor had decreased function and less NAP activity than the neutrophils isolated from the peripheral blood of the same donor.³⁶ Although the exact relationship between that observation and our results remains unclear, morphologically mature bone marrow neutrophils seem to be normal counterparts of the abnormal circulating neutrophils from the patients in this study and could be an experimental model for researching their pathological granulopoiesis. Interestingly, we initially observed that immature myeloid cells with low NAP activity from CML patients showed high NAP activity after incubation with rhG-CSF without sufficient morphological maturation. This phenomenon also suggests a discrepancy in neutrophil maturity between morphology and NAP activity. It seems, therefore, that NAP is not always an indicator of neutrophil maturity and G-CSF may be a specific activator of NAP, at least in these patients, although further study will be necessary to prove this hypothesis.

It is well known that patients with MDS frequently suffer from infections.³⁷⁻³⁹ Their susceptibility to infections is thought to be due to decreased numbers^{1,38,39} and impaired functions^{5,7-9} of their neutrophils because human neutrophils play critical roles in the host defense against invading microorganisms.^{40,41} In fact, Boogaerts et al showed that functional impairment of neutrophils correlated well with the observed susceptibility to infections in MDS patients.⁹ Patients with MDS are known to have a poor prognosis without evolution to acute leukemia,⁴² and one of the frequent causes of their deaths is infection.^{2,37-39} Therefore, neutrophil dysfunction seems to be an important clinical problem in these patients.

Using fMLP as a stimulant, we observed that neutrophil O_2^- production was frequently decreased in patients with MDS and that rhG-CSF was able to enhance this neutrophil function in these patients. O_2^- production is thought to be a reliable indicator of the oxygen-dependent microbicidal ability of neutrophils.^{40,41} Moreover, fMLP is the most potent chemotactic peptide that is closely related to the bacterial product.⁴³⁻⁴⁵ Our in vitro assay system is likely to reflect the physiological response of neutrophils in vivo when bacterial infection occurs, and the present results imply that rhG-CSF could enhance the bactericidal ability of neutrophils in MDS patients, which may result in increased resistance to infections.

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Hypermethylation of the 5' Region of the Calcitonin Gene Is a Property of Human Lymphoid and Acute Myeloid Malignancies

By Stephen B. Baylin, Eric R. Fearon, Bert Vogelstein, Andrée de Bustros, Saul J. Sharkis, Philip J. Burke, Stephen P. Staal, and Barry D. Nelkin

An abnormal increase in numbers of CCGG sites methylated in the 5' region of the human calcitonin (CT) gene occurred in tumor cell DNA samples from 90% (17 of 19) of patients with non-Hodgkin's T and B cell lymphoid neoplasms and in 95% (21 of 22) of tumor cell DNA samples from patients with acute nonlymphocytic leukemia (ANLL). The changes were not seen in patients with chronic myelogenous leukemia (0 of 9). The abnormal methylation patterns appear to be a property only of transformed or malignant cells since they were not found in DNA from nonneoplastic adult tissues including sperm, early myeloid progenitor cells, benign lymphoid hyperplasia, peripheral lymphocytes stimulated to divide, or early myeloid progeni-

tor cells (obtained by immunoaffinity using anti-My-10 antibody), but they did appear after Epstein-Barr virus transformation of lymphocytes. Moreover, during the course of therapy in patients with ANLL, the hypermethylation pattern reflects the presence of the leukemic clone even in normal-appearing granulocytes derived from this clone. The increased methylation of the CT gene may then provide an important molecular marker for biologic events in human cell transformation or tumor progression and may prove clinically useful in monitoring patients with lymphoid and acute myelogenous neoplasms.

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RECENTLY, there has been growing interest in the status of DNA methylation in human tumors. Studies to date have documented a relative hypomethylation of genomic DNA¹ and of specific genes²⁻⁴ in human tumors. In contrast, in the course of investigating the role of methylation in expression of the human calcitonin (CT) gene,^{5,6} we have obtained the unusual finding that, in over 80% of human small cell lung cancers, increased numbers of CCGG sites are methylated in the 5' region.⁵ The incidence of such a pattern was much less in other lung tumor types and in most other solid tumors examined.⁵ A significant exception to this pattern among non-small cell lung tumors, however, appeared to occur in human lymphomas. Five of 5 human lymphomas examined, 1 of T cell origin, and 4 of B cell origin had hypermethylation patterns similar to those seen in small cell lung carcinoma.⁵ These preliminary findings in the lymphomas and in one line of human promyelocytic leukemia cells have prompted us to study more extensively the methylation pattern of the CT gene in the major tumor types derived from the lymphohematopoietic system. We now report that hypermethylation of this gene is a general property of tumor cells in patients with human lymphoid malignancies and patients with acute (ANLL) but not chronic

nonlymphocytic leukemias. The CT gene hypermethylation may represent, from our studies to date of virally infected human lymphocytes and of DNA from early myeloid progenitor cells, an event specific for transformed or malignant cells rather than for stages of normal proliferation and differentiation within the lymphoid and myeloid series. In ANLL, the hypermethylation provides a molecular marker that monitors the presence of the active leukemic clone regardless of the state of morphological maturity of the tumor cells.

MATERIALS AND METHODS

Molecular probe. Our isolation of the 5' region-specific genomic probe for the CT gene has been previously described.⁵ In brief, the 1.7-kb probe, whose position is shown in Fig 1, was subcloned from a full-length genomic clone of the human CT gene⁷ and shown to be free of repeat sequences by a series of hybridization studies.⁵ All work involving recombinant DNA was performed in accordance with relevant National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules and guidelines of the Johns Hopkins Recombinant DNA Committee.

DNA hybridization studies. Hybridization patterns of restriction endonuclease digests of DNA from normal and neoplastic tissues or cells were obtained exactly as outlined in our previous studies.⁵ Preparation of DNA, restriction digests, electrophoresis of restricted DNA on 1% agarose gels, labeling of DNA probes, conditions of hybridization and filter washing, and autoradiogram exposure times were exactly as described.⁵ Approximately 5 µg of DNA was applied to each gel lane for all of the studies.

Tissues and cells. All patient materials were obtained under protocols approved by the Committee for Clinical Investigation for The Johns Hopkins Hospital. High-molecular weight DNA was extracted either from frozen tissues taken directly at the time of surgery, from peripheral blood cells, or from cells obtained during bone marrow aspiration as previously detailed.^{5,6}

The preparation and separation of leukemic blast cells from normal or neoplastic mature cells was accomplished by modifications of procedures of Boyum,^{8,9} as previously described.¹⁰ Briefly, peripheral blood cells or bone marrow cells were sedimented through 20 mL Histopaque (Sigma Chemical Co, St Louis) 1.077 gradients in 50-mL centrifuge tubes at 400 g for 40 minutes at 22°C. Tumor blast cells or normal mononucleated cells were harvested from the interface and polymorphonuclear leukocytes (granulocytes) from the pellet. As previously described¹⁰ each fraction of cells was extensively examined by light microscopy, and the leukemic blast

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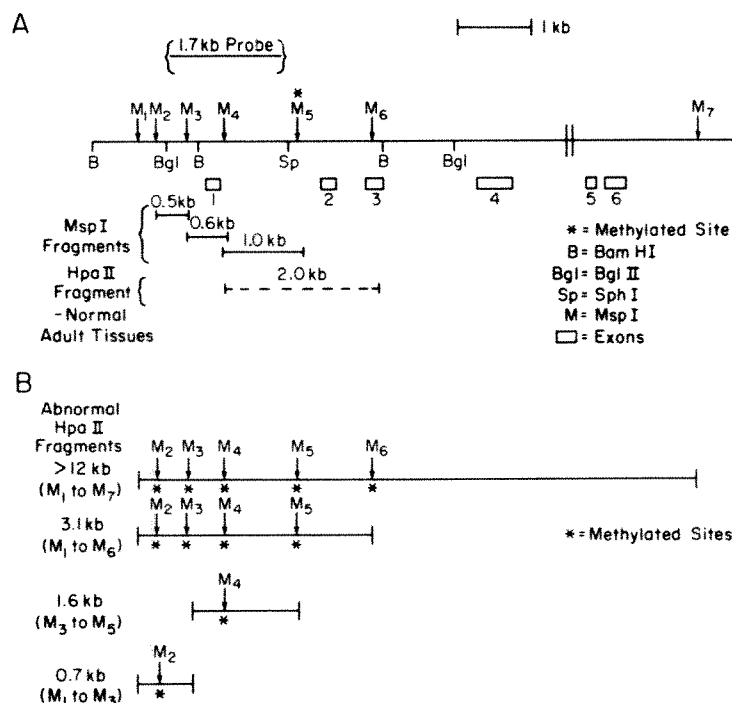


Fig 1. (A) An abbreviated schema of the structure of the human CT gene including a partial restriction map⁵ and designation of the seven *Msp*I sites (CCGG sequences, M₁ to M₇) involved with the methylation patterns discussed in the text. The position of the 5' region-specific genomic probe used in this study is also shown. The positions of the *Msp*I restriction fragments detected by this probe in genomic DNA are shown, and the *Hpa*II fragment of 2.0 kb, present in normal adult tissues because of methylation at site M₆ (asterisk), is also depicted. (B) Schematic of the CT gene and the CCGG sites and the positions of the characterized major fragments of abnormal methylation described in the text. The frequency for which each fragment was the predominant or codominant abnormal band in the lymphohematopoietic tumors studied is given in Table 1.

cells fractions contained >85% blast cells and the granulocyte fraction, >95% granulocytes.

Early myeloid progenitor cells were obtained by indirect immune adherence of normal human bone marrow cells by using the myeloid progenitor-specific anti-My-10 antibody^{11,12} as described previously.¹³ Briefly, adherent bone marrow cells were incubated with anti-My-10 hybridoma supernatant, washed in phosphate-buffered saline, and then incubated in a Petri dish coated with goat antimouse IgG. Adherent cells were collected, and DNA was extracted.

Epstein-Barr virus transformation of human lymphocytes. Forty milliliters of blood from a normal adult were treated exactly as described earlier for separation of mononucleated cells from granulocytes. Approximately 1×10^7 mononuclear cells were infected with 1×10^8 plaque-forming units of Epstein-Barr virus (EBV) and divided into multiple cultures in 1.0 mL of RPMI 1640 with antibiotics plus 2 μ g/mL cyclosporine and 15% fetal calf serum. Medium was changed one to two times per week until 6 to 7 weeks when the cells could be passaged. Cells were then maintained in 50 mL of the aforementioned medium.

RESULTS

Hypermethylation of the CT gene in lymphoid neoplasms. We show here that hypermethylation of CCGG sites in the CT gene is a general property of non-Hodgkin's lymphoid neoplasms. We previously mapped seven *Msp*I restriction sites in the human CT gene,⁵ five of which are in the 5' flanking or intron regions (Fig 1A). In human DNA, three *Msp*I restriction fragments (Fig 1A and Fig 2, lane 1) are detected by the 5' region-specific genomic probe shown

in Fig 1A. Digestion of DNA with *Hpa*II, an isoschizomer of *Msp*I that fails to cut CCGG sites if the internal cytosine is methylated,¹⁴ has identified one site, M₅ (Fig 1A), that is always at least partially methylated in DNA from a wide variety of adult normal tissues.⁵ Such digests (Fig 2, lane 2) are characterized by a variable reduction in intensity of the 1.0-kb band and appearance of a new 2.0-kb band spanning sites M₄ to M₆ (Fig 1A).

In contrast, *Hpa*II digests of genomic DNA from 17 of 19 lymphoid neoplasms examined, including 7 of 7 lymphomas (Fig 2, lanes 6 to 10) and 10 of 12 leukemias, (7 of 9 acute lymphoblastic, Fig 2, lanes 3, 4, 5, and 12; and 3 of 3 chronic lymphocytic leukemias, Fig 2, lane 11), revealed additional bands indicative of abnormalities of CT gene methylation patterns as compared with normal peripheral lymphocytes (Fig 2, lanes 16 to 19) and benign lymphoid hyperplasia (Fig 2, lanes 13 to 15). Lymphoid neoplasms of both T and B cell types had the abnormalities, thereby indicating that the unusual tumor methylation patterns are manifested in malignancies developing within both major differentiation pathways of the lymphoid system.

Three control studies indicate that methylation of the DNA sites examined accounts for the abnormal *Hpa*II digestion patterns in the lymphoid tumor cell DNA. First, for each of the abnormal patterns, digestion of the same DNA samples with *Msp*I yielded a normal restriction pattern, thereby confirming that the changes seen were not due to gene rearrangements or DNA polymorphisms. Second,

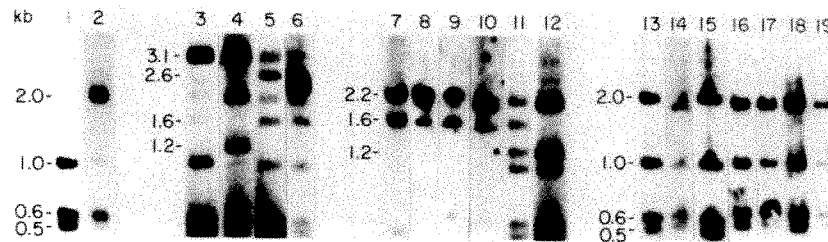


Fig 2. Hybridization patterns of restriction endonuclease digests of DNA from selected control tissues and lymphoid neoplasms and probed with the 5' region fragment⁵ of the human CT gene. Lane 1, *MspI* (10 U/ μ g) digestion of DNA from normal human liver. Lane 2, *HpaII* (4 U/ μ g) digestion of DNA from normal human thyroid showing the pattern typical for all adult normal tissues studied.⁵ Lanes 3 to 6, *HpaII* digests of DNA from tumor cells of patients with acute lymphoblastic leukemia (lanes 3 to 5) and B cell lymphoma (lane 6) that show the extensive pattern of CT gene methylation. Note the presence in all samples of abnormal bands at 3.1 (lanes 3 and 4) or 2.6 kb (lanes 5 and 6), and there are also abnormal bands of 2.2 kb (lane 6), 1.2 kb (lane 4), and 1.6 kb (lanes 5 and 6). Lanes 7 to 12, examples of *HpaII* digestion patterns found in DNA from tumors of the other patients with lymphoid neoplasms. The less extensive methylation pattern is seen with abnormal bands of 2.2 kb (lanes 7 to 9), 1.6 kb (lanes 7 to 10), and 1.2 kb (lanes 11 and 12). The diseases represented are human T-lymphotropic virus I-positive T lymphoma (lane 7), B cell lymphoma (lanes 7 to 10), chronic lymphocytic leukemia (lane 11), and acute lymphoblastic leukemia (lane 12). Lanes 13 to 15, *HpaII* digests of DNA from benign lymphoid hyperplasia from three individual patients. The patterns are identical to that in normal lymphocytes (lanes 16 to 19). Lanes 16 to 19, *HpaII* digestion patterns typical for DNA extracted from peripheral lymphocytes of ten normal individuals. The pattern is similar to that outlined for other normal adult tissues in Fig 1A and as shown in lane 2.

digestion of the tumor DNA with increasing quantities (two to 40 U/ μ g DNA) of *HpaII* failed to alter the patterns, thereby suggesting that the results were not due to partial digestion. Finally, partial *HpaII* digestion is further ruled out by the fact that hybridization of the same Southern blots with a probe for the human *c-myc* gene shows a complete *HpaII* digestion in both normal and tumor cell DNA.

The spectrum of hypermethylation patterns seen in the lymphoid tumors is summarized in Table 1 and involved predominantly the appearance, in 12 tumors, of unusual low molecular weight bands ranging from 2.2 to 1.2 kb (Fig 2, lanes 7 to 12), with seven of the 12 containing the 1.6-kb band (Fig 2, lanes 5 to 11). The genomic position of the 1.6-kb band (see Fig 1B) was previously determined in our studies of small cell lung cancer,⁵ and the positions of the 2.2- and 1.2-kb bands are not known. In five cases, a more extensive pattern of methylation was present (Fig 2, lanes 3

to 6), and this involved the appearance of a 3.1-kb band, also commonly seen in small cell lung cancer⁵ and spanning sites M_1 through M_6 (Fig 1B), and a 2.6-kb band the exact position of which is unknown.

Hypermethylation of the CT gene in nonlymphocytic leukemias. We have also established that CT gene hypermethylation is a property of ANLL. In our initial studies,² we found the extensive pattern (3.1-kb band) for hypermethylation of the CT gene in HL60, a cell line derived from human promyelocytic leukemia,¹⁵ a form of ANLL. We now find that 20 of 21 additional patients with ANLL had distinctly abnormal CT gene hypermethylation patterns in DNA from tumor blast cells taken from bone marrow at the time of disease presentation (Fig 3, lanes 1 to 11). Although the percentage of tumor cells bearing the abnormality may vary from virtually 100% (eg, lanes 1 and 2, Fig 3) to less than 50% (eg, the presence of both normal and abnormal bands in the other lanes of Fig 3), the methylation changes were detected easily in studies initially blinded to the clinical diagnosis in all 20 patients. These 20 leukemia samples spanned the entire range of immature myeloid differentiation patterns that constitute the subclassifications of ANLL.¹⁶ In contrast, DNA from tumor cells of nine of nine patients with chronic myelogenous leukemia, a much less aggressive disease upon initial presentation, contained a normal pattern of CT gene methylation (Fig 3, lanes 12 to 14). The pattern of CT gene methylation of ANLL tumor cells was also clearly different from the normal pattern in human bone marrow cells and normal peripheral blood granulocytes (Fig 3, lanes 15 to 20).

The distribution of hypermethylation patterns of the 20 abnormal DNAs from ANLL patients was different from that of the lymphoid tumors (Table 1). In ANLL tumors, 17 demonstrated, predominantly, the more extensive methylation pattern with fragments of 3.1 or 2.6 kb, whereas DNA from the other three patients had less extensive methylation abnormalities, with abnormal bands at 1.2 or 0.7 kb (position shown in Fig 1B) predominating.

Table 1. Distribution of the Dominant Abnormal *HpaII* Restriction Fragments in Tumors Studied

Abnormal Band (kb)	Lymphoid Neoplasms (N = 17)*		ANLL (N = 20)*	
	n	Percentage	n	Percentage
> 12	1 (ALL)	6	1	5
3.1	4 (1 lymphoma, 3 ALL)	25	11	55
2.6	2 (1 lymphoma, 1 ALL)	13	8	40
2.2	3 (2 lymphoma, 1 CLL)	19	1	5
1.6	7 (4 lymphoma, 1 ALL, 2 CLL)	44	0	0
1.2	2 (ALL)	13	2	10
0.7	0	0	1	5

Abbreviations: ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.

*Some tumors have codominant bands and, hence, are listed for two fragment sizes.



Fig 3. *HpaII* digestion patterns of DNA from tumor blast cells of patients with myelogenous leukemia and mature granulocytes from control individuals. Lanes 1 to 11, examples of patterns predominating in DNA from tumor blast cells of the 17 patients with the extensive pattern of CT gene methylation (see also Fig 1B). Among the abnormal patterns, either the 3.1-kb band alone predominates (lanes 1 to 6), the 3.1- and 2.6-kb bands codominate (lanes 7 and 8), or the 2.6-kb band dominates (lanes 9 to 11). Lanes 12 to 14, *HpaII* digests of tumor cell DNA of three of the nine patients studied with chronic myelogenous leukemia that showed a normal adult tissue pattern. Lanes 15 to 20, *HpaII* digests of DNA from normal granulocytes from six different normal individuals. The pattern is identical to that found in granulocytes from a total of 12 normal individuals and is typical of that found in other adult tissues examined (eg, Fig 2, lane 2).

Relationships of CT gene hypermethylation to cell differentiation, proliferation and transformation. The very high frequencies with which CT gene methylation abnormalities are found in certain neoplasms suggest that these events may be closely linked to either the transformation steps giving rise to most lymphomas and ANLLs or to changes occurring in tumor cell DNA during disease progression steps for these malignancies. To better place our findings into perspective regarding these possibilities, we have studied (a) whether the tumor-associated CT gene methylation patterns might reflect events ongoing during normal cellular differentiation in development or adult renewing cell systems, (b) whether the patterns seen might simply reflect changes in proliferative rates of parent cells for the tumors studied, or (c) whether the methylation patterns might represent de novo events occurring only in DNA from certain types of transformed or malignant cells. We have obtained data that strongly support the last possibility.

With respect to cell development and differentiation, it has been well established that changing patterns of gene methylation occur during embryonic development.¹⁷⁻¹⁹ Extensive methylation of genes is most common in sperm and in early embryonic cells.¹⁷⁻¹⁹ We examined *HpaII* digests of human sperm DNA from three individuals (Fig 4, lane 1) and of DNA from preparations of early myeloid (prepro-myelocytes or earlier) progenitor cells from three normal individuals (Fig 4, lane 2). These early myeloid cells are the presumed normal parent cells for ANLL leukemia cells and were obtained by flow cytometry sorting of normal human marrow cells tagged with the progenitor cell-specific monoclonal antibody My-10.¹¹⁻¹³ The pattern for both sperm and the myeloid progenitor cells was identical to those in normal adult tissues revealing methylation, particularly extensive in sperm, only of CCGG site M₅. These results in human sperm cells and in early myeloid lineage cells in the bone marrow strongly suggest that the tumor-associated changes in CT gene methylation do not reflect patterns that are found during normal cell development or steps in adult cellular differentiation.

Increased cell proliferation per se does not appear to explain the observed patterns of tumor-associated CT gene methylation. As previously mentioned, we compared *HpaII* digestion patterns of DNA extracted from malignant lymphoid cells with those found in benign hyperplastic lymphoid cells (Fig 2, lanes 13 to 15). The patterns for the hyperplastic cells were identical to those found in normal adult tissues and did not resemble those found in the lymphoid malignancies studied. Also, we have failed to find the methylation changes in human peripheral T lymphocytes chronically stimulated to divide with interleukin-2.

Our next series of findings firmly suggests that the CT gene hypermethylation seen in some tumors arises during the transformation process and subsequently reflects the tumor activity of the transformed clone. We first studied CT gene methylation early (2 months) after EBV infection of human peripheral B lymphocytes. At this time, immortal cell lines are just obtained that undergo constant proliferation but that are not usually tumorigenic.²⁰ In six of nine total lines established from two normal individuals (eg, Fig 4, lane 3) CT gene hypermethylation was present, and the patterns represented the entire range of those abnormalities seen in lymphomas and lymphoid leukemias.

Relationships of CT gene hypermethylation to clonal evolution of ANLL, tumor cell differentiation, and treatment response. We next studied, during the course of disease, methylation patterns as a function of clonality in cells of patients with ANLL. ANLL, like many malignancies, develops as a clonal cellular disorder.^{21,22} The presence of a single leukemic clone was mapped in five patients by DNA hybridization studies of the X-linked hypoxanthine phosphoribosyl transferase (HPRT) gene in females whose normal tissues had a polymorphism for this gene²³ or by hybridization patterns for genes specifically associated with documented abnormal chromosomal patterns in the leukemic cells of these females.¹⁰ Specifically, in the patients who were not polymorphic for the HPRT gene, clonality of cells was determined by either DNA hybridization studies mapping polymorphisms of the carbonic anhydrase II gene in a patient with trisomy of chromosome 8 in tumor cells or mapping the loss of an allele for the procollagen $\alpha 2(I)$ gene in a patient with the loss of one copy of chromosome 7 from tumor cells.¹⁰

In all five patients, the extensive pattern of CT gene hypermethylation (abnormal 3.1- or 2.6-kb bands) was present in all clonal tumor blast cell samples at the time of initial disease presentation (Fig 4, lanes 4, 11, 14, 17, and 19). In

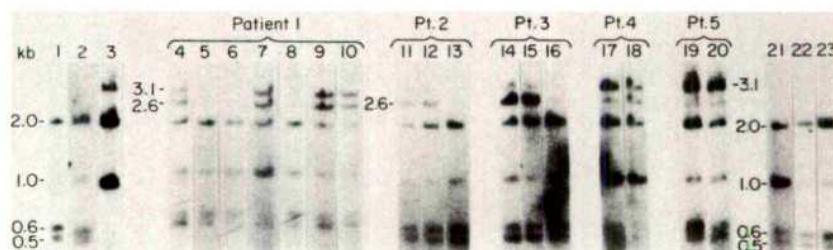


Fig 4. *HpaII* digests of DNA from sperm, myeloid progenitor cells, EBV-transformed cells, and cells of eight patients with myeloid leukemia at various times during treatment of the disease. The clonality of the tumor cell populations in the patients described (studies in lanes 4 to 23) was always 80% to 100% as judged by the hybridization procedures outlined in the text and by Fearon et al¹⁰ and Vogelstein et al.²³ Lane 1, example of pattern in sperm DNA from three individuals. Lane 2, example of pattern seen in DNA from enriched population of My-10 bearing early myeloid progenitor cells in three separate preparations of marrow cells from three different individuals. Lane 3, example of the CT gene hypermethylation seen in DNA from six of nine EBV early transformed B lymphocytes. Lanes 4 to 10, Samples from patient 1. Lane 4, clonal blast cells at disease presentation, July 3, 1984; lane 5, polyclonal granulocytes at the time of first therapy-induced remission, October 4, 1984; lane 6, polyclonal mononuclear cells, October 4, 1984; lane 7, clonal blast cells at time of first disease recurrence, January 26, 1985; lane 8, polyclonal granulocytes at time of second therapy-induced disease remission, February 21, 1985; lane 9, clonal blast cells at time of second disease recurrence, April 4, 1985; lane 10, clonal granulocytes at disease recurrence, April 4, 1985. Lanes 11 to 13, samples from patient 2. Lane 11, clonal blast cells at time of disease presentation, March 25, 1985; lane 12, clonal granulocytes at disease presentation, March 25, 1985; lane 13, polyclonal granulocytes at time of therapy-induced remission August 8, 1985. Lanes 14 to 16, samples from patient 3. Lane 14, clonal blast cells at time of disease presentation, February 21, 1985; lane 15, clonal granulocytes at disease presentation, February 21, 1985; lane 16, polyclonal granulocytes at time of therapy-induced remission, April 25, 1985. Lanes 17 to 20, Samples from patients 4 and 5. Lane 17, clonal blast cells at time of disease presentation in patient 4; lane 18, clonal granulocytes at disease presentation in patient 4; lane 19, clonal blast cells at the time of disease presentation in patient 5; lane 20, clonal granulocytes at disease presentation in patient 5. Lanes 21 to 23, Patterns in DNA from clonal granulocytes of three different patients with ANLL in long-term (0.5 to 3 years) remission.

three patients, the methylation pattern returned to normal in both polyclonal granulocytes (Fig 4, lanes 5 and 8, patient 1; lane 13, patient 2; lane 16, patient 3) and mononuclear cells (Fig 4, lane 6, patient 1) during periods of polyclonal remission after therapy, a status indicating either complete abolition of the tumor clone or its eradication below detectable limits. In the same patients, either initially or during periods of recurrence of the disease after therapy, the abnormal pattern of methylation was again present, not only in tumor blast cells but, importantly, also in morphologically normal-appearing granulocytes that were shown from clonal analysis by gene hybridization testing to arise from the tumor clone (Fig 4, patient 1, lane 10; patient 2, lane 12; patient 3, lane 15). In another two female patients, we also detected abnormal methylation patterns in mature-appearing granulocytes derived from the leukemic clone (Fig 4, lane 18, patient 4; lane 20, patient 5). These results in the aforementioned five patients indicate a close linkage between the hypermethylation pattern of the CT gene and the derivation of both blast cells and more differentiated appearing myeloid cells from the malignant leukemic clone.

Finally, we obtained further evidence that the abnormal CT gene methylation reflects the activity of the leukemic clone rather than resulting solely from clonal cell selection. We studied DNA from marrow-derived cells of three patients who were in long-term remission (0.5 to 3 years) after successful induction chemotherapy. In each of these individuals, however, the mature-appearing granulocytes were clonal rather than polyclonal by gene hybridization studies. No tumor blast cells were present, and the patients were clinically well, thus suggesting the presence of either an inactive leukemic clone, a preleukemic clone of some type, or a bone marrow repopulated after therapy by a clone of normal cells. In these granulocytes, the CT gene methylation

pattern was identical to that for normal adult tissues in each case (Fig 4, lanes 21 to 23).

DISCUSSION

Hypermethylation of CCGG sites in the 5' region of the CT gene appears closely linked to the transformed and malignant phenotype of cells in the major forms of human lymphohematopoietic tumors. The findings not only provide a potential molecular marker for key stages of transformation during the development of these cancers but also for monitoring the clinical status of patients with these malignancies. This may prove especially so for ANLL, a tumor for which no consistent molecular abnormality has been identified previously. CT gene methylation patterns appear to mark the activity of the abnormal leukemic clone in ANLL and may help predict, during therapy, which patients have had complete eradication of tumor cells and which patients will have sustained remissions of disease.

The exact mechanism accounting for the methylation changes observed has not been determined, but the events seem closely tied, temporally, to transformation or tumor progression events in the cell systems studied. It is of interest that, despite previous observations of DNA hypomethylation in human tumors, Kautiainen and Jones have recently reported that the enzyme activities responsible for methylation of eukaryotic DNA can be markedly increased in human and rodent tumor cells.²⁴ Both maintenance and de novo DNA methyl transferase activities were especially high in leukemic cells.²⁴ Most important for our current observations, these authors found no correlation between overall levels of DNA methylation and levels of the methylating enzymes, and they speculated that some specific gene sequences might reflect qualitative changes secondary to the

increased DNA methylating capacity of the tumor cells.²⁴ Our findings for the CT gene may be the first example of such a qualitative change in the genome.

The functional implication of CT gene hypermethylation for the process of neoplasia remains to be determined. Although this gene has no defined role in determining the phenotype of normal and neoplastic cells in bone marrow, other genes active in the differentiation process of such cells could be involved in the abnormal methylation process we have observed. Increased methylation in CG-rich 5' regions of genes can potentially inhibit gene transcription.^{25,26} Such

reduced gene activity could contribute to the abnormalities of growth and differentiation common to neoplastic cells, and our findings should stimulate investigation of the methylation status of other genes in lymphoid and myeloid tumors.

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Normal Cellular Counterparts of B Cell Chronic Lymphocytic Leukemia

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In an attempt to compare B cell chronic lymphocytic leukemia (B-CLL) with its normal cellular counterpart, the cell surface phenotype of 100 cases of B-CLL was determined by using a panel of monoclonal antibodies (MoAbs) directed against B cell-restricted and -associated antigens. The majority of B-CLL cells expressed Ia, B4 (CD19), B1 (CD20), B2 (CD21), surface immunoglobulin (slg), and T1 (CD5) but lacked C3b (CD35) receptors. In contrast, the overwhelming majority of small unstimulated B cells expressed Ia, B4, B1, B2, slg, and C3b receptors but lacked detectable T1. Small numbers of weakly slg+ cells could be identified in peripheral blood and tonsil that coexpressed the B1 and T1 antigens. Approximately 16% of fetal splenocytes coexpressed B1, T1, weak slg, B2, and Ia but lacked C3b receptors and therefore closely resembled most B-CLL cells. With the phenotypic differences between the majority of small unstimulated B cells and B-CLL cells, we examined normal in vitro activated B cells

and B-CLL cells for the expression of B cell-restricted and -associated activation antigens. Of 20 cases examined, virtually all expressed B5, and approximately 50% of the cases expressed interleukin-2 receptors (IL-2R) and Blast-1. Normal B cells were activated with either anti-Ig or 12-O-tetradecanoylphorbol- β -acetate (TPA) and then were examined for coexpression of B1, T1, and the B cell activation antigens B5 and IL-2R. Only cells activated with TPA coexpressed B1 and T1 as well as B5 and IL-2R. B cells activated with either anti-Ig or TPA proliferated in the presence of IL-2, whereas B-CLL cells did not, although they all expressed the identical 60-kilodalton proteins by immunoprecipitation. These studies are consistent with the notion that B-CLL resembles several minor subpopulations of normal B cells including a population of B cells that are activated in vitro directly through the protein kinase C pathway.

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GREATER THAN 95% of chronic lymphocytic leukemias (CLL) are of B cell lineage by virtue of their expression of monoclonal cell surface immunoglobulin (slg),¹⁻³ clonally rearranged immunoglobulin genes,^{4,5} and the expression of B cell-restricted cell surface antigens (Ags).⁶⁻¹¹ Although the B cell origin of CLL is generally accepted, the subset of normal B cells from which B cell CLL (B-CLL) is derived remains unresolved. The difficulty in identifying the normal cellular counterpart of B-CLL is based upon its unique cell surface phenotype and functional properties. B-CLL cells differ from morphologically identical unstimulated small B lymphocytes in that they lack C3b receptors (CD35),^{12,13} express the 67-kilodalton (kD) T cell-associated T1 (CD5) Ag,¹⁴⁻²⁰ and form rosettes with mouse erythrocytes (MRBC-R).²¹⁻²³ Moreover, B-CLL cells demonstrate variable responsiveness to mitogens that activate most normal small B lymphocytes.²⁴⁻²⁷ These phenotypic and functional differences suggest that B-CLL appears to be derived from a unique subset of B lymphocytes.

Previous attempts to identify the subset of B cells from which B-CLL cells arise have been largely based upon morphology and analysis of cell surface phenotype. Until recently, it was generally believed that B-CLL cells were derived from small unstimulated peripheral blood B lymphocytes since they were morphologically identical and weakly coexpressed IgM and IgD. Moreover, both populations also expressed HLA class II molecules as well as other B cell-restricted Ags including B4 (CD19),¹¹ B1 (CD20),⁶ and B2 (CD21),⁹ the C3d/Epstein-Barr virus (EBV) receptor. However, the coexpression of T1 and MRBC-R and B-CLL cells led investigators to abandon the peripheral blood B cell in search of another B cell subset that might express these structures. Several studies have now identified subpopulations of normal B cells that resemble B-CLL cells.^{28,29} For example, a very small subpopulation of slg-positive B cells (2% to 3%) was identified at the edge of the germinal center of the secondary follicle in tonsil and lymph node material that coexpressed T1 and formed MRBC-R. Similarly, T1 + B cells have been identified in human fetal lymph node and spleen tissue, and these cells appear to be a major subpopulation of fetal B cells. These studies provided evidence for one or more subpopulations of normal B cells that phenotypically resembled B-CLL cells.

In the present report, we have attempted to extend these studies by relating B-CLL cells to subpopulations of normal B cells. In the studies to be reported later, we will demonstrate that small populations of B cells can be identified in adult peripheral blood, adult lymphoid tissue, and fetal lymphoid tissue that coexpress the B1 and T1 Ags. Moreover, B-CLL cells express several cell surface Ags that are expressed on activated B cells but not resting B cells, thereby suggesting the B-CLL cells may be the neoplastic counterpart of a subpopulation of activated B cells. Further evidence for this notion is derived from the observation that 12-O-tetradecanoylphorbol- β -acetate (TPA) but not anti-Ig can induce small unstimulated B lymphocytes to express B1 and T1 as well as B cell activation Ags.

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MATERIALS AND METHODS

Adult Human Tissues Cells

Normal spleen, tonsil, and bone marrow were obtained after appropriate Human Protections Committee validation and informed consent. Normal spleen was obtained from operative specimens of patients not known to have any systemic or malignant disease. Tonsil cells were obtained at the time of routine tonsillectomy. Nucleated bone marrow cells were recovered by Ficoll-Hypaque centrifugation. Single-cell suspensions of spleen and tonsil tissue were prepared by dissolution in Hanks' balanced salt solution (HBSS) with forceps and scissors and extrusion through a stainless steel mesh. Mononuclear cells isolated by Ficoll-Hypaque density gradient centrifugation were enriched for B cells by E rosetting to deplete T cells. Cells were either used fresh or cryopreserved in 10% dimethyl sulfoxide and 20% fetal calf serum (FCS) at -196°C in the vapor phase of liquid nitrogen until the time of study. Cells were recovered at viabilities of 70% to 90%.

Isolation of Peripheral Blood Cells

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteer donors by Ficoll-Hypaque density sedimentation (Pharmacia Fine Chemicals, Piscataway, NJ). Unfractionated mononuclear cells were separated into sIg⁺ and sIg⁻ populations by Sephadex G-200 (Pharmacia) anti-F(ab')₂ chromatography with modifications designed to minimize monocyte retention by columns as previously described.⁶ T cells were recovered by E rosetting the sIg⁻ population with 5% sheep erythrocytes. Normal human monocytes were removed by adherence to plastic culture dishes.

Fetal Tissues

Fetal tissues were obtained within one hour of prostaglandin-induced abortion. All patients undergoing therapeutic abortion had last menstrual periods and diagnostic ultrasound imaging that suggested that the fetal age was less than 24 weeks. To standardize gestational age, age determination postmortem was determined by crown-rump length and fetal foot length. Procurement of tissue was approved by the Brigham and Women's Hospital Committee on Human Subjects in Research, and informed consent was obtained from all patients undergoing therapeutic abortion.

Fetal bone marrow and single-cell suspensions of spleen and liver were prepared as previously described.³⁰

Patients Samples

Tumor cells were obtained from peripheral blood of previously untreated patients with CLL from Brigham and Women's Hospital and Dana-Farber Cancer Institute after appropriate Human Protection Committee validation and informed consent. The diagnosis was based on a total peripheral blood lymphocyte count of $15 \times 10^9/\text{L}$ and an infiltration of well-differentiated lymphocytes in the bone marrow. B cell lineage was established by the presence of monoclonal sIg or the pan-B cell antigen B1 as determined by indirect immunofluorescence with the use of anti- κ , λ , IgG, IgM, IgD, and B1 monoclonal antibodies (MoAbs).

MoAbs

The preparation and characterization of MoAbs used in this study have been described. The Ags to which these antibodies are directed are summarized and referenced in Table 1. All MoAbs used in this study were ascites fluid at saturated binding concentrations.

Table 1. Expression of Lineage-Restricted and -Associated Ags

Ag	CD Designation	Normal Cellular Reactivity	Molecular Weight (kD)	Reference
B1	20	pan-B	35	6
B2	21	Limited B	140	9
B4	19	pan-B	95	11
Ia		pan-B	29, 34	31
sIg		Limited B	IgM-900 IgG-150 IgD-150	31
B5		Activated B	75	32
Blast-1		Activated B	45	33
Blast-2	23	Activated B	45	34
BB1		Activated B	37	35
IL-2R	25	Activated T, B	55	36
T9		Proliferating cells/ nonlineage re- stricted	90	37
C3bR	35	B, RBC, monocyte, granulocyte	220	38
Mo2	14	Monocyte		39
T1	5	T, thymocytes, sub- set of B	67	14-20
T11	2	T, thymocyte	50	40

Fluorescent Staining

Indirect. Cells were prepared in 10% pooled AB serum in HBSS; when the cells had been incubated with anti-Ig coupled to beads, they were incubated in human serum for one hour at 37°C to remove the beads from the cell surface. Aliquots of 10^6 cells were incubated with each antibody (generally a 1/100 to 1/400 dilution of ascites) for 30 minutes at 4°C . After washing, the cells were incubated with a 1/50 dilution of fluorescein-conjugated goat anti-mouse Ig antibody (Tago Inc, Burlingame, CA) for 20 minutes at 4°C . The cells were washed and were either analyzed fresh or were fixed in 1% formaldehyde for subsequent analysis.⁶ A reaction was considered positive when greater than 10% of the test cells were more fluorescent than the number of cells positive with isotype-identical control ascites. For each sample, a quantitative assessment of the number of positive cells was made (number of cells reactive with the test antibody minus the number of cells reactive with unreactive isotype-identical monoclonal antibody/10,000 total cells tested).

Direct and dual-fluorescence staining. Directly fluorescein conjugated MoAbs were prepared as previously described.⁶ For directly phycoerythrin (PE) conjugated MoAbs, 1.0 mg of protein A-purified anti-B1, anti-interleukin-2 receptor (IL-2R) antibody, or a euglobulin precipitate purification of anti-B5 were reacted with 0.5 mg of R-phycoerythrin (pyridyldisulfide derivative) as described elsewhere.⁴¹

MoAbs were biotinylated by standard techniques.¹¹ The specificity of each of these conjugated antibodies was tested on appropriately reactive normal tissues and cell lines and found to be identical with that of the unconjugated antibodies. To define the percentage of cells that expressed the B1 and T1 Ags within each cellular population, the number of cells with positive fluorescence was compared with the number of cells stained with negative control antibody out of a total of 500 to 1,000 cells counted on a fluorescent microscope (Carl Zeiss, West Germany). Cells with two or more discrete positive clumps per cell or cells with clear-cut peripheral rims were scored as positive. To enumerate the number of B1+ cells that coexpressed the T1 Ag, 100 B1+ cells were counted, and the percentage of these cells expressing T1 was determined. To characterize the B1+ F(ab')₂ column nonadherent population, the number

of cells expressing the B1 and T1 Ags was first defined. The number of directly fluoresceinated B1+ cells coexpressing other cell surface determinants was next enumerated by counting the number of B1+ cells that stained with a second directly biotinylated MoAb and were developed with avidin Texas red dye.¹¹ By using these techniques, it was possible to accurately identify the number of B1+ cells that coexpressed several other cell surface determinants. All flow cytometric analysis was performed on either an EPICS V or C cell sorter (Coulter Electronics, Hialeah, FL). Dual-fluorescent-stained cells were analyzed after initial calibration of the machine with cells stained with each individual fluorochrome-labeled antibody and with other controls as outlined previously.¹¹ When beads were present in the cell suspensions, they could be excluded from the analysis by setting the forward-angle light scatter gates to exclude particles less than 5 μ m in diameter. MRBC-R were enumerated by the method previously described.²²

B Cell Cultures

Large-scale B cell cultures. The E- fraction of splenic mononuclear cells was further enriched for B cells by two treatments with the MoAbs anti-Mo1, anti-Mo2, anti-T4, and anti-T8 followed by rabbit complement to deplete all but B cells from the spleen cell suspensions. These highly enriched B cells were cultured for two days at 1.5×10^6 /mL in RPMI 1640 supplemented with 10% FCS, 2 mmol/L glutamine, and 1 mmol/L sodium pyruvate in tissue culture flasks (Corning Glass Works, Corning, NY), with either affinity-purified F(ab')₂ rabbit antihuman Ig coupled to Affigel 702 beads (anti-Ig) (Bio-Rad, Richmond, CA) as previously described⁴² or TPA (Sigma Chemical Co, St Louis) used at a final concentration of 10 ng/mL, and 10^{-4} mol/L 2-mercaptoethanol.

Microcultures. Highly enriched splenic B cells were prepared from the E- fraction of splenic mononuclear cells by anti-T cell and antimonocyte MoAb and complement lysis. CLL cells were highly purified by a similar depletion of T cells and monocytes. Cells were cultured in 96-well, round-bottom microtiter trays (Costar, Cambridge, MA) at 50,000 per well. Anti-Ig beads and recombinant IL-2 (rIL-2) (a gift of the Biogen Corp, Boston) were added to yield a final volume (per well) of 200 μ L. Previous studies have demonstrated that maximal stimulation of normal B cell ³H-thymidine incorporation with RI-L2 is at 200 U/mL.⁴³ T cell conditioned medium (TCM) was prepared as previously described.⁴² TPA was used at a final concentration of 10 ng/mL.

Thymidine Uptake Assay

Thymidine uptake was used as an index of mitogenic activity. Microcultures were pulsed with 0.2 μ Ci of ³H-thymidine (Amersham Corp, Eastbourne, England) per well and were harvested 15 hours later. Dried filters were counted on a Packard Tri-carb scintillation counter (Downers Grove, IL).

Labeling of Cells With Radioisotopes

A modification of the lactoperoxidase iodination technique was used. The labeled cells included IL-2R+ CLLs that were further enriched for B cells by lysis with anti-T cell and antimonocyte antibodies and complement. T cells were activated with anti-T11₂ and -T11₃ antibodies for three days.⁴⁴ Highly enriched splenic B cells were activated with TPA (10 ng/mL) for two days. The iodinated cells were washed twice with cold phosphate-buffered saline and lysed on ice with cell lysis buffer (50 mmol/L Tris-HCl, 0.4 mol/L NaCl, 1% Triton X-100, 2 mmol/L phenylmethylsulfonylfluoride, 5 mmol/L EDTA, 50 mmol/L iodoacetamide, pH 8). After 30 minutes the lysate was centrifuged at 800 g for ten minutes to remove unlysed cells, nuclei, and other insoluble material. The supernatant was frozen at -80°C until analyzed.

Immunoprecipitation

Cell supernatants and lysates were centrifuged at 100,000 g for 15 minutes and then precleared four times: twice for one hour at 4°C with either Sansorbin (for Ig precipitations) or Pansorbin (Calbiochem-Behring Corp, La Jolla, CA), once with Sepharose 4B beads, and once with an irrelevant antibody conjugated to Sepharose 4B. The precleared lysates were mixed with anti-IL-2R antibody conjugated to Sepharose 4B beads. The mixtures were held on ice for 2 hours and then washed four times with 1% Triton X-100 and 1% sodium deoxycholate in 12 mmol/L sodium phosphate, 5 mmol/L EDTA, 5 mmol/L ethylene glycol tetraacetic acid (EGTA), and 1 mmol/L NaF, pH 7.4 (RIPA buffer). Precipitates were analyzed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) (10%). Ig precipitates were performed in an identical manner except that in the precipitation of the precleared supernatant we used antihuman Ig antibody bound to protein A—Sepharose 4B (Pharmacia, Uppsala, Sweden).

RESULTS

Expression of B Cell-Associated Ags on B-CLL Cells

Tumor cells from 100 patients with the clinical and morphological features of B-CLL were tested for reactivity with a panel of MoAbs. Tumor cells in all cases were of B cell derivation by the expression of B1 and B4 (Table 2). All but five cases expressed T1. Ninety of 100 cases expressed the EBV/C3d receptor B2.⁴⁵⁻⁴⁷ The two major phenotypic subgroups were defined by the expression of sIg and the receptor for C3b (C3bR). Monoclonal sIg was not detectable on the tumor cells of 21 patients. Only 19 of the 100 patients with B-CLL expressed the C3b receptor. The overwhelming majority of B-CLL cells coexpressed Ia, B1, B4, T1, and B2. Three major subgroups could be identified by the examination of expression of sIg and C3bR: (a) sIg+, C3bR- (n = 51); (b) sIg+, C3bR+ (n = 17); and (c) sIg-, C3bR- (n = 15).

Examination of the intensity of Ag expression was also undertaken (Table 2). Ia and B1 were strongly expressed on all tumor cells (Fig 1). The B2 and B4 Ags were less intensely expressed but were clearly positive on most tumor cells (60% to 80%) in the neoplastic population. Cell sIg, T1, and C3bR were much less intensely expressed on the cell

Table 2. B Cell Ag Expression of CLL

No. of Patients Expressing Ag	Ag Intensity						
	Ia	B1	B2	B4	sIg	T1	C3bR
51	+++	+++ / ++	++	++	+	+/++	
17	+++	+++ / ++	++	++	+	+/++	+
15	+++	+++ / ++	++	++		+/++	
2	+++	+++ / ++	++	++		+/++	+
6	+++	+++ / ++		++	+	+/++	
4	+++	+++ / ++		++		+/++	
5	+++	+++ / ++	++	++	+		

The degree of positivity was qualitatively assessed by flow cytometry. Blank space, no detectable reactivity over background; +, weak (sIgM on peripheral blood B cells, Fig 1); ++, moderate (B2 on peripheral blood B cells, Fig 1); +++, strongest (B1 on peripheral blood B cells, Fig 1). The range of percent positive cells for each antigen was as follows: Ia, 65% to 85%; B1, 70% to 90%; B2, 50% to 70%; B4, 60%-80%; T1, 60% to 80%; C3bR, 20% to 40%. The percentage of cells expressing sIg was difficult to quantitate due to weak expression.

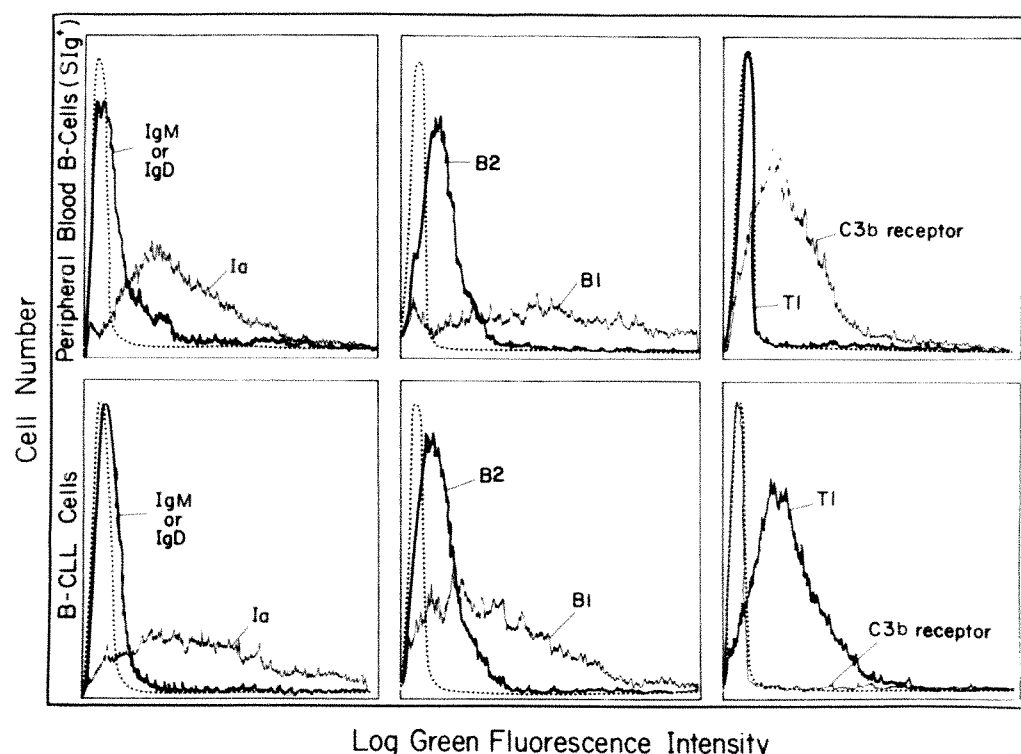


Fig 1. Fluorescence-activated cell sorter histogram of the reactivity of anti-IgM, -IgD, -Ia, -B2, -B1, -T1, and C3bR with normal peripheral blood B cells (top) and B-CLL cells (bottom). Background fluorescence was obtained by incubating the cells with isotype-identical unreactive MoAb and developing with goat antimouse Ig FITC.

surface. When compared with small resting B cells isolated from peripheral blood or spleen tissue that express Ia, B4, B1, B2, sIg, and C3bR, the intensity of sIg and C3bR was significantly less intense on B-CLL cells.

Identification of T1 Ag-Positive B Cells in Normal Lymphoid Populations

With the observation that most B-CLL cells express Ia, B4, B1, sIg, and T1, we attempted to identify normal B lymphocytes that expressed the B-CLL phenotype. Mononuclear cells isolated from peripheral blood, tonsil, and bone marrow were examined for the presence of cells that coexpressed B1 and T1 Ags. In these experiments, the number of B1- and T1-positive cells was first enumerated, and then the number of cells coexpressing T1 was determined (Table 3). In the PBMC fraction, few if any B1+T1+ cells were identified. When T cells were removed by E rosetting, the resulting E- fraction demonstrated approximately 3% of cells that coexpressed B1 and T1, whereas no B1+ cells coexpressed T3. B cells enriched by anti-F(ab')₂ column chromatography isolated from peripheral blood and tonsil tissue were 70% B1+, and approximately 10% to 20% of these B1+ cells coexpressed T1. Of great interest was the observation that approximately 5% of the column-nonadherent sIg- cells isolated from either peripheral blood or tonsil tissue expressed B1. These "sIg-" cells were passed over the column a second time in an attempt to remove all cells that expressed sIg; nonetheless, 5% of the sIg- or very weakly sIg+ population remained B1+. Approximately 25% of these column-nonadherent B1+ cells were T1+, which

supports the notion that a population of normal B cells exists that coexpresses B1, T1, and very faint sIg. Of note was the observation that in normal adult bone marrow, none of the B1+ cells were T1+.

Since very small numbers of B cells isolated from adult lymphoid organs coexpressed B1 and T1, fetal tissues were also examined for the presence of cells that coexpressed B1 and T1. As seen in Table 3, approximately 40% of fetal splenocytes expressed B1. In contrast to adult B1+ cells,

Table 3. Identification of B1+T1+ Cells Within Normal Adult Lymphoid Populations and Fetal Lymphoid Organs

Cell Population	No. of Tests	B1 (%)	T1 (%)	T3 (%)	Percentage of the B1-Positive Cells Coexpressing T1
Adult					
PBMC	3	6 ± 3	60 ± 10	67 ± 8	1 ± 1
E-	3	25 ± 9	17 ± 7	12 ± 6	3 ± 2
E+	3	1 ± 1	95 ± 11	96 ± 8	0
PB sIg+	3	80 ± 10	8 ± 5	4 ± 3	10 ± 4
PB sIg-	3	5 ± 2	65 ± 10	60 ± 5	28 ± 7
Tonsil sIg+	3	70 ± 10	20 ± 5	8 ± 6	12 ± 5
Tonsil sIg-	3	6 ± 3	84 ± 9	81 ± 8	27 ± 9
Bone marrow	3	3 ± 2	8 ± 3	7 ± 5	0
Fetal					
Spleen	10	40 ± 8	40 ± 10	ND	40 ± 5
Bone marrow	4	15 ± 6	5 ± 3	ND	1 ± 1
Liver	3	10 ± 5	3 ± 2	ND	1 ± 1

Abbreviation: ND, not determined.

approximately 40% of these B1+ fetal spleen cells coexpressed T1. Similar to adult bone marrow, very few fetal bone marrow B1+ cells expressed T1. Moreover, fetal liver cells that expressed B1 did not appear to express T1.

Isolation and Phenotypic Characterization of Anti-F(ab')₂ Column-Nonadherent B1-Positive Fetal Splenocytes

To define a population or populations of normal B cells that correspond to B-CLL cells, fetal splenocytes were isolated that expressed B1, T1, and faint sIg. Fetal splenocytes were fractionated into the sIg+ and sIg- fractions. The column-adherent cells were 80% sIg+ with strong sIg expression. In contrast, cells that were nonadherent to the column contained approximately 10% to 20% B1+ cells. These cells, like most B-CLL cells, expressed such faint sIg that they readily passed through the anti-F(ab')₂ column.

Due to the phenotypic similarity of these anti-F(ab')₂ column-nonadherent B1+ fetal splenocytes to B-CLL cells, further characterization of these cells was undertaken by using dual fluorescence. Table 4 depicts three experiments examining the cell surface phenotype of these cells. The overwhelming majority of these B1+ cells coexpressed IgM, IgD, Ia, B2, and T1, but very few, if any, expressed T3. Although column-nonadherent B1-positive fetal splenocytes were T1+, the majority of these cells lacked C3bR. The relative intensity of Ag expression on these cells was comparable to that seen with B-CLL cells.

Expression of B Cell Activation Ags on Activated B Cells and B-CLL Cells

We and others have previously observed that B-CLL cells express B cell activation Ags. In an attempt to compare B-CLL cells with the stages of normal B cell activation, unstimulated normal splenic B cells were first cultured with the B cell-specific mitogen anti-Ig, and a temporal sequence of antigenic changes was evaluated. As shown in previous studies, anti-Ig-activated B cells lose sIgD, B2, and B3 (CD22).⁴⁸ Moreover, they acquire a number of Ags not expressed on resting B cells. Very few, if any, unstimulated B cells expressed the B cell-restricted activation Ags B5, Blast-1, Blast-2 (CD23), and BB1 or the B cell-associated activation Ags T9, the transferrin receptor, and IL-2R (CD25). After activation, however, all were expressed and increased for approximately three days. On day 1, although small numbers of cells (10% to 15%) expressed activation Ags, only B5 and IL-2R were consistently expressed. By day 2, between 30% and 55% of the cells expressed B5, IL-2R, T9, and BB1, whereas the number of cells expressing Blast-1 and Blast-2 were consistently less (10% to 25%). By day 3,

the number of cells expressing each Ag was maximal, with 40% to 75% of cells expressing B5, IL-2R, and BB1, and 75% to 90% of cells were T9+. Blast-1 and Blast-2 were unchanged from the level of expression seen at two days. By six days of culture, significantly fewer cells expressed any of the activation Ags (10% to 40%), although none had returned to the background level. The maximal expression of activation Ags closely paralleled our previous studies of ³H-thymidine incorporation of in vitro-activated B cells.⁴²

Tumor cells from 20 patients with B-CLL were examined for the expression of these activation Ags. These cases all had total peripheral lymphocyte counts of greater than 15,000/ μ L. Eleven of 20 of these cases expressed Ia, B1, B4, T1, B2, and sIg and lacked C3bR. Four additional cases coexpressed the aforementioned Ags but in addition coexpressed C3bR. Of the B cell activation Ags examined, the B5 Ag was most frequently expressed (19 of 20) and was present on 60% to 90% of cells within a given population (Table 5). Blast-1 and IL-2R were expressed on approximately 50% of the cases, with 40% to 60% and 20% to 50% of cells within a given population expressing the Ags, respectively. Blast-1 and IL-2R were generally present on the same cases. Blast-2 was expressed on six of 20 cases, and BB1 was infrequently expressed (with 20% to 50% and 40% to 70% of cells Ag-positive, respectively). T9 was not detected on any of the tumor samples examined.

The intensity of Ag expression of three of these activation Ags was different from their expression on normal anti-Ig-activated B cells. Both the IL-2R, Blast-1, and BB1 were less strongly expressed, and fewer Ag-positive cells were present on B-CLL cells (20% to 40%) than on normal anti-Ig-activated B cells (40% to 70%). In contrast, although the Ag intensity of B5 on CLL cells was similar to normal activated B cells, B5 was expressed on essentially all the cells in each case of B-CLL, whereas normal anti-Ig-activated B cells are more heterogeneous in their expression of B5, with 40%-60% of cells within a population expressing this Ag.

Induction of T1 Ag on Normal B Cells After Activation With TPA

With the demonstration that B-CLL cells express T1 and B cell activation Ags, we then attempted to induce unstimulated normal B cells to express the B-CLL cell phenotype. It has been recently observed that the T1 Ag could be induced on normal peripheral blood B cells with TPA.⁴⁹ We therefore activated highly purified resting splenic B cells with either anti-Ig or TPA in an attempt to induce the coexpression of T1 and the B cell activation Ags B5 and IL-2R. For the 48 hours of culture with anti-Ig, recovery of B cells was between 70% and 80%. Recovery of B cells after culture with TPA

Table 4. Phenotypic Characterization of the B1-Positive F(ab')₂ Column-Nonadherent Cells Isolated From Fetal Spleen

	Ag						
	sIgM	sIgD	Ia	B2	T1	T3	C3b Receptor
Percentage of B1+ cells coexpressing Ags	80 $\pm$ 12	70 $\pm$ 17	96 $\pm$ 4	67 $\pm$ 15	72 $\pm$ 12	2 $\pm$ 1	15 $\pm$ 5
Relative fluorescence intensity	+	+	+++	+	++	ND	++

Table 5. Activation Ag Expression of CLL

No. of Patients Expressing Ag	Ag Intensity					
	B5	IL-2R	Blast-1	Blast-2	BB-1	T9
7	++	+	+			
5	++					
3	++			+		
2	++	+	+		+	
1	++	+		+		
1	++		+	+		
1			+	+		

The range of percent positive cells for each Ag was as follows: B5, 60% to 90%; IL-2R, 20% to 50%; Blast-1, 40% to 60%; Blast-2, 20% to 50%; BB-1, 40% to 70%.

was approximately 50%, probably due to residual adherence of cells to the culture flasks.

Before stimulation, a population of highly enriched splenic B cells that were greater than 90% B1+ contained less than 1% T cells and monocytes as assessed by the expression of T11 and Mo2, respectively (Table 6). This population contained about 5% B5+ and IL-2R+ cells and less than 1% T1+ cells. As seen in Table 6, 34% of the TPA-activated cells expressed T1, with 78% and 66% of the cells expressing B5 and IL-2R, respectively. In this experiment, Blast-1 was expressed on 27%, Blast-2 on 38%, T9 on 31%, and BB-1 on 54%, respectively. These TPA-activated cells failed to form MRBC-R. In contrast, splenic B cells cultured for one to five days with anti-Ig also expressed B5 and IL-2R but failed to express T1. The absence of contaminating T cells and monocytes together with the high percentage of cells expressing the B cell-restricted Ag B1 support the notion that the B cells were the population undergoing the observed antigenic changes and were not indirectly due to effects of TPA on T cells and monocytes. The recovery of adequate cell numbers after stimulation also supports the view that we were observing antigenic changes and not selecting minor subpopulations of B cells.

To further clarify the phenotype of TPA-activated B cells, cells were examined before and after culture with TPA by using dual-laser flow cytometric analysis. Splenic B cells were dual fluorochrome labeled with fluorescein isothiocyanate (FITC)-conjugated anti-T1 and PE conjugated to either anti-B1, anti-B5, or anti-IL-2R. As seen in Fig 2A, no cells expressing either T1 alone or T1 and B1 were detectable before culture with TPA. However, after two days of culture with TPA, T1+B1+ cells were detected as well as cells expressing only B1 (Fig 2B). Similarly, within the population of TPA-activated cells, subpopulations expressing B5 and T1 (Fig 2C) were observed as well as IL-2R and T1 (Fig 2D). Moreover, virtually all T1+ cells were B5+ and IL-2R+. These studies suggest that the B cells that are induced to express T1 also coexpress B5 and IL-2R and that

Table 6. Percentage of Cells Expressing Ag (Ag Intensity)

	B1	T1	B5	IL-2R	T11	Mo2
Control, day 0	90 (++++)	1	6 (+/-)	6 (+/-)	1	1
TPA, day 2	91 (++++)	34 (+/++)	78 (++)	66 (+)	1	1
Anti-Ig day 2	85 (++++)	1	46 (++)	31 (+)	1	1

Results are one of three representative experiments.

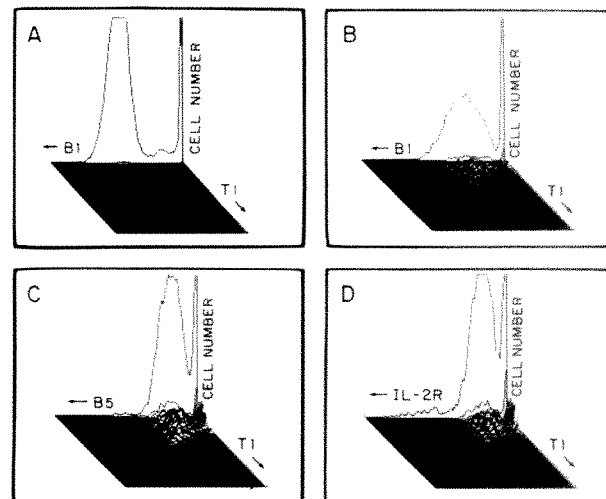


Fig 2. Highly enriched splenic B cells examined before (A) and after stimulation with TPA, 10 ng/mL, for two days (B, C, D). Cells were stained with directly fluoresceinated anti-T1 (anti-T1F) and directly PE conjugated anti-B1 (panels A and B), anti-T1F and directly PE conjugated anti-B5 (C), and anti-T1F and directly PE conjugated anti-IL-2R (D). Cells were then examined by dual-laser flow cytometric analysis.

B1+T1+B5+IL-2R+ B cells are subset of normal activated lymphocytes.

In an attempt to definitely demonstrate that B-CLL cells express IL-2R, peripheral blood lymphocytes from two patients with B-CLL that were greater than 90% B1+ were further enriched for B cells by anti-T cell and antimonocyte MoAb and complement lysis. B-CLL cells were precipitated with either anti-IL-2R, anti-B4, or anti-B2. TPA-activated normal B cells were precipitated with anti-IL-2R. Activated T cells were immunoprecipitated with anti-IL-2R and 4B4, which identifies a T cell-associated Ag. The immunoprecipitates were analyzed by 10% SDS-PAGE. Autoradiography of the gel (Fig 3) showed that a single band corresponding to a protein with a mol wt of 60,000 was precipitated from T

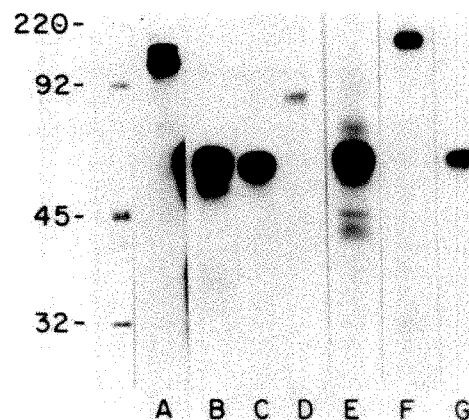


Fig 3. SDS-PAGE (10%) of ^{125} I-labeled normal T cell blasts (lanes A and B immunoprecipitated with 4B4 [T cell-associated Ag] and anti-IL-2R, respectively); CLL cells from two patients (lanes C, D, E, and F immunoprecipitated with anti-IL-2R [lanes C and E], anti-B4 [lane D], and anti-B2 [lane F]; and normal TPA-activated B cells immunoprecipitated with anti-IL-2R (lane G).

cell blasts (lane B), cells from two patients with B-CLL (lanes C and E), and normal activated B cells (lane G). These studies suggested that the 60,000-dalton structures immunoprecipitated from normal activated T cells, TPA-activated B cells, and B-CLLs were identical.

Differential Effect of Recombinant IL-2 on IL-2R-Positive B-CLL Cells and In Vitro-Activated B Cells

In an attempt to functionally compare B-CLL cells with in vitro-activated normal B cells, we examined IL-2R+ B-CLL cells and normal activated B cells for proliferation in response to recombinant IL-2 (rIL-2). Previous studies have demonstrated that normal anti-Ig-activated B cells that express IL-2R proliferate in response to rIL-2.⁴³ The peak of proliferation occurs at about three days of in vitro culture and decreases to baseline by approximately six days. Normal resting splenic B cells were cultured with media alone, rIL-2, anti-Ig, TPA, anti-Ig and rIL-2, or TPA and rIL-2. As seen in Table 7, resting B cells could be induced to proliferate with anti-Ig or TPA. In contrast, rIL-2 alone induced minimal proliferation, probably secondary to small numbers of in vivo-activated B cells. The addition of rIL-2 to anti-Ig or TPA led to a consistent augmentation of between two- and fivefold. We next examined tumor cells from five patients with B-CLL that were all IL-2R+ (3 cases were sIg+, C3bR-, 1 was sIg-, C3b-, and 1 was sIg+, C3b+). In contrast to resting B cells, tumor cells from these five patients with B-CLL did not proliferate to either rIL-2, anti-Ig, or a combination of them (Table 7) when examined at three days. One patient (CLL 2) had a minimal proliferative response to TPA with some augmentation by rIL-2. By six days of culture, ³H-thymidine incorporation of both normal B cells and CLL cells was at the background level. Previous investigators have noted proliferation of CLL cells in response to anti-Ig and TCM. The substitution of TCM for rIL-2 yielded results similar to those seen with rIL-2 (data not shown). These studies suggested that although some B cell CLLs express IL-2R they fail to proliferate when cultured in the presence of this growth factor.

DISCUSSION

In the present report, we have examined the cell surface phenotype of 100 cases of B-CLL and attempted to identify normal B cells that might represent its normal cellular counterpart. The overwhelming majority of B-CLL cells expressed Ia, B4 (CD19), B1 (CD20), B2 (CD21), sIg, and T1 (CD5) but lacked C3bR (CD35). Although, unstimulated small B cells expressed Ia, B4, B1, B2, and sIg, in

addition they expressed C3bR and lacked detectable T1 expression. B cells isolated from normal lymphoid populations were then examined for the expression of Ags that were present on B-CLL cells. Small numbers of weakly sIg+ cells expressing B1 and T1 were observed in adult peripheral blood and tonsil tissue but not bone marrow. Greater numbers of these cells could be identified in fetal spleen material. Weakly sIg+, B1+ fetal splenocytes were further examined and were shown to coexpress T1, sIgM, sIgD, Ia, and B2 but lacked C3bR and thus closely resembled B-CLL cells. Considering the phenotypic differences between small unstimulated B cells and B-CLL cells, we examined B-CLL cells and normal in vitro-activated B cells for the expression of Ags expressed on activated but not resting B cells. Of 20 cases of B-CLL examined, virtually all expressed the B cell-restricted activation Ag B5 and approximately 50% expressed Blast-1 and IL-2R (CD25). Unstimulated small splenic B cells were then stimulated with anti-Ig or TPA. Although both anti-Ig and TPA induced B5 and IL-2R expression on normal stimulated B cells, only TPA-activated cells coexpressed T1. With the observation that TPA-activated B cells and some B-CLL cells expressed receptors for IL-2, we attempted to determine whether these cells could respond to IL-2 and whether the receptor on B-CLL cells was identical to that found on activated T and B cells. Although B-CLL cells and activated B cells express IL-2R, only in vitro-activated B cells proliferated to rIL-2. Immunoprecipitation demonstrated that the identical 60-kD cell surface proteins were expressed on B-CLL cells and activated T and B cells, thereby demonstrating that both cells express IL-2R. These results are consistent with the notion that small numbers of normal B cells either isolated from fetal or adult lymphoid tissues or induced by TPA activation appear to be candidates for the normal cellular counterpart of the B-CLL cell.

Classically the activation of B lymphocytes occurs by the cross-linking of cell sIg by either Ag or anti-Ig.⁵⁰ This initial event induces resting B cells to increase pools of intracellular calcium and inositol triphosphate. Subsequently these cells then synthesize RNA, increase in size, and finally become competent to respond to a variety of B cell growth factors (BCGF) including low- and high-molecular weight BCGF, IL-2, and γ -interferon.⁵¹ Alternatively, EBV, TPA, and C3d as well as MoAbs directed against Bp35 (CD20) also induce several of these events as they trigger resting B cells, probably through alternative pathways of activation, to leave the G₀ phase of the cell cycle.^{52,53} For example, TPA stimulates B cells via direct activation of protein kinase C without increases in intracellular calcium. After activation with

Table 7. Response of Normal B Cells and CLL Cells to rIL-2

	³ H-Thymidine Incorporation (cpm)					
	Media	rIL-2	TPA	Anti-Ig	rIL-2 + Anti-Ig	TPA + rIL-2
Normal B cells	476 ± 67	2524 ± 266	15,192 ± 2,028	5,204 ± 702	22,430 ± 5,178	37,466 ± 2,597
CLL 1	123 ± 27	246 ± 53	218 ± 97	138 ± 51	176 ± 105	89 ± 17
CLL 2	114 ± 43	146 ± 29	812 ± 176	378 ± 181	205 ± 23	1,212 ± 245
CLL 3	111 ± 12	176 ± 23	131 ± 89	179 ± 48	176 ± 15	264 ± 29
CLL 4	153 ± 23	186 ± 24	98 ± 42	254 ± 26	189 ± 57	175 ± 37
CLL 5	102 ± 23	211 ± 53	326 ± 98	136 ± 13	166 ± 29	344 ± 36

anti-Ig, a distinct sequence of antigenic changes has been consistently observed.⁴² The earliest activation Ags can be detected by 24 hours, with peak expression observed by 72 hours. By six days most activation Ags are either lost or significantly decreased. The phenotype of the B-CLL cell with its expression of Ia, sIg, B4, B1, and B2 as well as the activation Ags B5, Blast-1, Blast-2, and IL-2R suggests that this cell might be the neoplastic counterpart of a major subpopulation of activated B cells. However, B-CLL cells express T1, thereby demonstrating that this cell does not correspond to a population of anti-Ig-activated normal B cells. The observation that TPA-stimulated B cells that coexpress B1 and T1 as well as the B cell activation Ags B5 and IL-2R suggests that direct activation via the protein kinase C pathway induces a minor subpopulation of activated B cells that are phenotypically similar to most B-CLL cells.

Although TPA-activated B cells resemble B-CLL cells, they do not appear to be an identical population. Although virtually all B-CLL cells express receptors for MRBCs, resting B cells activated with either anti-Ig or TPA do not express detectable numbers of MRBC-R. Moreover, although B-CLL cells express IL-2R, they do not proliferate to IL-2. Preliminary studies from our laboratory suggest that T1+, TPA-activated normal B cells that express IL-2R proliferate in response to IL-2. Our data are in contrast to several previous reports where responsiveness to IL-2 has been seen in B-CLL cells.⁵⁴⁻⁵⁷ Whether this is due to patient selection or to the presence of very small numbers of contaminating normal activated T cells is unclear. This lack of responsiveness may be due to a predominance of low-affinity IL-2R on B-CLL cells.⁵⁸ A recent report suggests that the high-affinity IL-2R may consist of two subunits of 55- and 70-kD.⁵⁹ B-CLL cells as well as hairy cell leukemia cells and neoplastic pre-B cells that also express IL-2R but fail to proliferate to IL-2 may lack the 70-kD subunit that appears to be necessary for proliferation.⁶⁰⁻⁶² The differences in MRBC-R and responsiveness to IL-2 suggests that the B-CLL normal cellular counterpart may be a subpopulation of the B1+T1+ population.

The cell surface phenotype of B-CLL cells appears to be remarkably homogeneous, with approximately 90% of tumors expressing Ia, B4, B1, B2, and T1. Heterogeneity was seen in the expression of sIg and C3bR and to a lesser extent B2. The demonstration of this heterogeneity of B-CLL cells

may be reflective of the various normal T1+ B cell populations from which B-CLL cells are derived or states of activation of the B1+T1+ population. Several investigators have identified normal B cells that resemble B-CLL cells. In situ studies have suggested that these cells are present in very small numbers at the periphery of the germinal center of normal adult lymph node²⁸ whereas larger numbers of these cells could be identified in fetal spleen and lymph node,²⁷ in the peripheral blood of patients after allogeneic bone marrow transplantation,⁶³ and in peripheral blood of patients with rheumatoid arthritis.⁶⁴ In murine systems, Ly1+ B cells, which may be the counterpart of human T1+ B cells, secrete autoantibodies and therefore may represent activated autoreactive B cells.⁶⁵ In the present study, we identified B1+T1+ cells in adult peripheral blood and tonsil tissue but not bone marrow. Because T1+ B cells are a major population of fetal splenocytes, we used this tissue as a source of this subset of B cells. We observed that these B1+ cells weakly expressed both sIgM and sIgD as well as Ia, B2, and T1 but lacked T3 and that only 15% of these cells expressed C3bR. In preliminary experiments, small numbers of these cells coexpress B5 but do not appear to express IL-2R. This immunophenotype as well as the intensity of Ag expression on these cells was remarkably similar to that observed for the majority of B-CLL cells.

In summary, the present study provides evidence that B-CLL is derived from minor populations of B1+T1+ lymphocytes. Moreover, the observation that this phenotype could be induced via the direct activation of protein kinase C is consistent with the hypothesis that subpopulations of normal B cells may be activated via distinct pathways of activation. Future studies will be directed toward identifying the subset of unstimulated B cells that are induced to become T1+. Studies comparing T1+ normal B cells with B-CLL cells and anti-Ig-activated normal B cells (T1-) for their responses to various growth and differentiation factors may also lend insight into the defect in humoral immunity of B-CLL.

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Pseudo-Bernard-Soulier Syndrome: Thrombocytopenia Caused by Autoantibody to Platelet Glycoprotein Ib

By Dana V. Devine, Mark S. Currie, Wendell F. Rosse, and Charles S. Greenberg

The Bernard-Soulier syndrome is an inherited bleeding disorder that is due to a deficiency in platelet glycoprotein Ib. Bernard-Soulier platelets fail to agglutinate in response to ristocetin despite normal levels of factor VIII: von Willebrand factor. We report a patient who developed severe refractory thrombocytopenia postsurgically while receiving procainamide therapy. Thrombocytopenia was immune mediated since the patient's platelets bore high levels of antiplatelet antibody. Radioimmunoprecipitation studies demonstrated that the autoantibodies had specificity for

platelet glycoproteins Ib and V as well as platelet HLA. The patient's plasma as well as purified immunoglobulin G completely inhibited the ristocetin-induced aggregation of normal platelets but did not inhibit adenosine diphosphate-induced aggregation. The laboratory studies revealed that this patient suffered from antibody-mediated thrombocytopenia with unusual characteristics that we have called pseudo-Bernard-Soulier syndrome.

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THE BERNARD-SOULIER SYNDROME (BSS) is an inherited bleeding disorder characterized by moderate thrombocytopenia, prolonged bleeding time, giant platelets on the peripheral blood film, normal platelet aggregation in response to adenosine diphosphate (ADP), epinephrine, and collagen, but no aggregation in response to ristocetin. These platelets are deficient in the membrane glycoprotein (GP) Ib,^{1,2} which is the binding site for factor VIII: von Willebrand factor.³ The membrane proteins GP V and GP IX have also been reported to be deficient in BSS platelets.⁴

We report a patient who developed refractory thrombocytopenia and an IgG autoantibody that selectively inhibited ristocetin-induced platelet aggregation. Biochemical analysis of purified immunoglobulin demonstrated autoantibodies to platelet GP Ib, GP V, and nonpolymorphic determinants on HLA molecules.

MATERIALS AND METHODS

Case report. The patient was a 76-year-old white female who was admitted to Duke University Hospital for open heart surgery to replace the mitral and tricuspid valves. Before the open heart surgery, she had a cardiac catheterization via a right femoral approach from which she developed a right groin hematoma. Preoperative coagulation studies demonstrated a normal prothrombin time (PT) and partial thromboplastin time (PTT) and a platelet count of 105,000/ μ L. The patient had received procainamide for a year before surgery to treat ventricular tachycardia. During surgery procainamide was continued as an intravenous infusion. The patient received 7 units of packed red cells and 10 units of platelets in the operating room. Postoperative coagulation testing revealed a PT of 12 seconds (normal, 9 to 10 seconds), PTT of 39 seconds (normal, 23 to 34 seconds), thrombin clotting time (TCT) of 22.5 seconds

(control, 20), fibrin degradation products (FDP) level less than 10 μ g/mL, and fibrinogen level of 330 mg/dL. The patient developed petechiae and suffered from repeated nose bleeds. Her platelet count progressively declined after surgery, with a nadir of 6,000/ μ L occurring on the eighth postoperative day. At this time, her WBC count was 4,700/ μ L, with 60% granulocytes, 26% lymphocytes, and 14% monocytes. Repeat coagulation studies performed on the ninth postoperative day showed a PT of 11 seconds, PTT of 35 seconds, TCT of 23.7 seconds, fibrinogen at 360 mg/dL, FDP less than 10 μ g/mL, and no fibrin monomer. The patient received several platelet transfusions from which she never obtained an increment in her platelet count. In accordance with institutional guidelines, the patient was advised of procedures and attendant risks and gave informed consent for a bone marrow biopsy. The bone marrow biopsy sample revealed the presence of megakaryocytes in the marrow. Her stained peripheral blood smear did not demonstrate giant platelets. Prednisone therapy was started; however, her thrombocytopenia never corrected. The patient subsequently developed a pneumonia that was unresponsive to parenteral antibiotics. She died on the 35th postoperative day.

Methods. Blood samples were anticoagulated in sodium citrate (1 part 3.2% sodium citrate:9 parts blood) or were allowed to clot in glass tubes. IgG was purified from normal or patient serum by ammonium sulfate precipitation and diethyl aminoethyl cellulose chromatography as described.⁵ IgG was purified from patient serum that was obtained 4 weeks after procainamide therapy had been discontinued.

Either direct or indirect radioimmunoassays for platelet-bound IgG were performed as described⁵ with mouse monoclonal anti-IgG (Fc) (Miles, Elkhart, IN). For indirect assays, washed normal platelets at 5×10^8 /mL in phosphate-buffered saline (PBS) containing 0.015 mol/L EDTA (PBS-EDTA) were reacted with an equal volume of patient or autologous serum for 30 minutes at room temperature (RT), washed twice in PBS-EDTA, and incubated with ¹²⁵I-labeled monoclonal anti-IgG. Bound radiolabel was separated from unbound by centrifugation through phthalate esters.⁵ Indirect assays for C3 deposition were performed by incubating normal platelets washed into veronal-buffered saline with an equal volume of patient serum that had been diluted 1:9 with normal serum. After three washes in PBS-EDTA, the platelets were reacted with ¹²⁵I-labeled monoclonal anti-C3d (Cytotech, San Diego). A similar indirect binding assay was performed for antigranulocyte antibodies.⁶ Microlymphocytotoxicity assays were performed by using the method of Amos et al.⁷

Immunoprecipitation studies were carried out with ¹²⁵I-labeled washed normal platelets as described.⁸ Briefly, surface-labeled platelets were solubilized in buffer containing the nonionic detergent Nonidet P-40 and reacted with the patient serum or autologous serum. Immunoprecipitations of GP Ib were performed by using monoclonal antibody to GP Ib (6D1) or monoclonal antibody that

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precipitates the GP Ib/GP IX complex. Antigen-antibody complexes were precipitated by incubation with formalin-fixed protein A-bearing *Staphylococcus aureus*. For complexes containing monoclonal antibody, the bacteria were coated with goat antimouse immunoglobulin antibody (Tago Diagnostics, Inc, Burlingame, CA). The antigen-antibody complexes were removed from the bacteria by boiling in electrophoresis sample buffer. The proteins were separated under nonreducing conditions by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5 to 15% acrylamide gradient in the resolving gel. The gel was dried, and autoradiography was performed.

For sequential immunoprecipitation experiments, the platelet lysate was treated four times with monoclonal antibodies to GP Ib and GP Ib/IX to remove GP Ib from the platelet lysate. Control lysate was treated four times with monoclonal antibody P3, which does not react with platelet proteins. Both lysates were then reacted with patient serum as described earlier. Densitometric scans of the autoradiographs were made with a Hoefer GS-300 transmittance/reflectance scanning densitometer (San Francisco).

Platelet aggregation studies were performed with a Bio-Data aggregometer according to the manufacturer's recommendations. Ristocetin cofactor activity aggregation assays were performed by using both fresh, gel-filtered platelets and formalin-fixed, washed platelets from normal donors. Some ristocetin aggregation assays were performed with platelets that were incubated with normal donor or patient IgG. Four hundred microliters of gel-filtered normal platelets were incubated with 100 μ L IgG at 4.1 or 1.0 mg/mL for five minutes at RT before the addition of ristocetin (1.5 mg/mL final concentration) and pooled normal plasma. ADP (0.4 μ mol/L final concentration) aggregation was performed by mixing normal donor platelet-rich plasma with patient platelet-poor plasma or pooled platelet-poor plasma from normal donors.

RESULTS

Initial platelet aggregation studies looking for antiheparin antibody in this patient's plasma were confounded by the fact that the patient's platelet-poor plasma lysed the test platelets as determined both by visual inspection of the assay mixture and the platelet count. However, if the patient plasma was heat inactivated for 30 minutes at 56°C, no lysis was observed. By indirect radioimmunoassay, both IgG (ranging from 9,000 to 12,000 molecules/platelet) and C3 (8,742 molecules/platelet) were detected on the surface of normal platelets reacted with the patient's serum. The patient's IgG bound equally well to PI^{AI}-positive and PI^{AI}-negative platelets (10,733 and 11,694 molecules/platelet, respectively), thereby excluding a diagnosis of posttransfusion purpura.

Platelet immunoprecipitation experiments showed that the patient's serum contained antibodies that precipitated protein bands with a relative mobility corresponding to platelet GP Ib (mol wt = 168 kilodaltons [kD]), the heavy chain (43 kD) and light chain 14 kD) of HLA, and a glycoprotein of 79 kD (Fig 1). When monoclonal antibodies reactive with GP Ib were used to deplete platelet lysate of GP Ib, the patient's serum no longer precipitated the 168-kD band from the lysate (Fig 2).

Lymphocytotoxicity analysis of the patient's serum uncovered anti-HLA antibody(s) with broad specificity that lysed all target lymphocytes on a 30-donor test panel. Antibodies in the patient's serum also bound to and activated complement on paraformaldehyde-fixed granulocytes at twice nor-

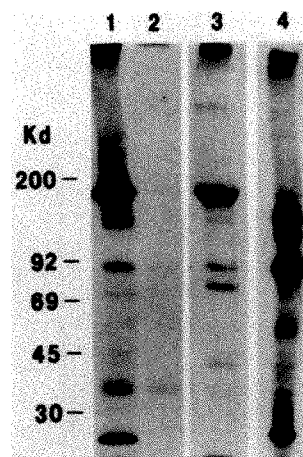


Fig 1. Immunoprecipitation of radiolabeled platelet glycoproteins by patient serum and monoclonal antibody to platelet GP Ib. Solubilized radiolabeled platelets were reacted with monoclonal antibody to GP Ib (lane 1), autologous plasma (lane 2), or the patient's plasma (lane 3). The patient's plasma precipitated proteins corresponding to GP Ib (mol wt, 168 kD), HLA (heavy-chain mol wt, 43 kD; and light-chain mol wt, 14 kD), and GP V (mol wt 79 kD). Lane 4 contains the lysate from the surface-labeled platelets. GP III (mol wt, 89 kD) was a contaminating protein in lanes 1 to 3. Monoclonal antibody to GP Ib³ was the kind gift of Dr Barry Coller.

mal levels as measured by anti-IgG and anti-C3 radioimmunoassay. The specificity of the antigranulocyte antibody was not determined. It must be noted, however, that the patient was not neutropenic.

The platelet aggregation studies demonstrated that the patient's plasma inhibited the ristocetin cofactor activity of pooled normal plasma when tested with either fresh washed platelets or formalin-fixed platelets. IgG purified from the patient's serum caused the same inhibition. IgG from normal

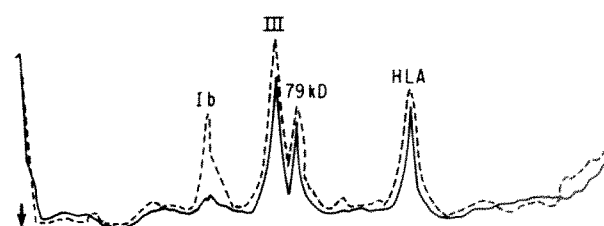


Fig 2. Sequential immunoprecipitation of GP Ib. GP Ib was removed from solubilized radiolabeled platelets by precipitation with monoclonal antibody to GP Ib/IX as described in the text. Precipitations were performed four times, at which time GP Ib was no longer detected in autoradiographs of the precipitates. Control lysates were treated with monoclonal antibody that does not react with platelet proteins (P3). The platelet lysates were then reacted with patient serum and antibody-antigen complexes electrophoresed in SDS-PAGE. The gels were subjected to autoradiography, then scanned with a densitometer. Patient serum precipitated bands corresponding to GP Ib, the heavy chain of HLA, and a protein of 79 kD from lysate treated with P3 (dashed line). Patient serum failed to precipitate the band corresponding to GP Ib from lysate that no longer contained GP Ib (solid line). The arrows at the left and right edges of the tracing mark the top and bottom of the gel, respectively. As in the experiment shown in Fig 1, GP III was a contaminating protein in the immunoprecipitations.

Table 1. Patient's Laboratory Data

	Patient	Normal Range
Factor VIII antigen	150%	50%-150%
Ristocetin cofactor activity	150%	50%-150%
Antiplatelet antibody (molecules/platelet)	10,286	<1,200
Posttransfusion purpura screen	Negative	
Circulating immune complexes	Elevated	
Total serum complement (CH50)	33.8	38-46
Serum protein electrophoresis	Normal	
Fluorescent antinuclear antibody	1:2,560, speckled pattern, specificity for antiribonucleoprotein	
Platelet aggregation studies		
Normal platelets incubated with patient plasma and ADP	57% aggregation (control, 62%; normal range, 45%-85%)	
Normal platelets incubated with patient plasma and ristocetin	1% aggregation (normal plasma control, 97%)	
Normal platelets incubated with patient IgG (4.1 mg/mL) and ristocetin	3% aggregation (buffer control, 100%)	
Normal platelets incubated with patient IgG (1.0 mg/mL) and ristocetin	69% aggregation (buffer control, 100%)	

donor serum did not affect the ristocetin aggregation assay, nor did plasma from patients with polyspecific anti-HLA antibodies that lysed all donor cells in the lymphocytotoxicity panel. Effective inhibition of ristocetin cofactor activity required a brief (ten-minute) incubation of test platelets with patient IgG before the addition of ristocetin and pooled normal plasma. When the IgG preparation was added to the cuvette last, only 14% inhibition was achieved. No inhibition of ADP-induced aggregation by the patient plasma was seen. The relevant laboratory findings are summarized in Table 1.

DISCUSSION

One of the diagnostic criteria for BSS is the inability of BSS platelets to agglutinate in response to ristocetin in the presence of a normal factor VIII: von Willebrand factor level. The failure to agglutinate to ristocetin is due to the absence of GP Ib, the binding site for von Willebrand factor, on the platelet membrane.¹⁻³ The patient reported here produced an antibody that interacted with GP Ib and inhibited ristocetin agglutination. The detection of this antibody was hampered because the patient also had anti-HLA antibody. In addition, the antiplatelet antibodies she made activated sufficient complement to bring about platelet lysis. Complement-mediated destruction of her own and transfused platelets may have contributed to the thrombocytopenia since her plasma lysed platelets *in vitro*. The anti-GP Ib antibody was

responsible for the thrombocytopenia since despite having anti-HLA antibodies the patient was not leukopenic.

One other case has been documented of a patient who developed autoantibodies that caused "acquired" BSS. The patient reported by Stricker et al⁹ differed significantly from the one reported here owing to the fact that their patient maintained a normal platelet count and that, with one exception, the patient did not demonstrate clinical bleeding.

The antiplatelet autoantibodies in this patient's serum contained reactivity not only with platelet HLA and GP Ib, but also with a 77-kD membrane protein that has a relative mobility similar to that of GP V. GP V has been reported to be deficient in the platelets of patients with BSS.⁴ The spatial relationship of GP Ib and GP V in the platelet membrane is not known. It is possible that the two molecules are closely associated and the antibody produced by this patient precipitated them as a complex.

Development of anti-GP Ib antibodies has been described in three of 106 patients with chronic idiopathic thrombocytopenic purpura (ITP),¹⁰ although no platelet function studies were performed with those antibodies. Interestingly, Szatkowski et al¹¹ reported a patient with ITP who had an anti-GP Ib antibody that significantly enhanced ristocetin aggregation and ADP aggregation but did not, by itself, induce platelet aggregation. It is possible that individual anti-GP Ib antibodies recognize different determinants and may either enhance or inhibit agglutination or cause thrombocytopenia.

The patient reported here had received procainamide therapy; therefore drug-induced thrombocytopenia must be considered. The antiplatelet antibody was not drug dependent, however, since samples of serum obtained 30 days after discontinuation of procainamide treatment contained antibody that precipitated GP Ib in the absence of the drug. The induction of autoantibodies in procainamide-treated patients is well documented.^{12,13} Although these autoantibodies are predominantly against histones,¹⁴ ribonucleoprotein,¹⁵ and denatured DNA,¹⁶ antilymphocyte antibodies¹⁷ and rheumatoid factor¹⁸ have also been reported. The patient reported here had antilymphocyte antibodies that appeared to be directed, in part, against HLA antigens. Whether procainamide therapy induced the production of anti-HLA and platelet-specific autoantibodies cannot be determined in this case.

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A Colony Assay for In Vitro Transformation by Human T Cell Leukemia Viruses Type I and Type II

By Mordechai Aboud, David W. Golde, Noelle Bersch, Joseph D. Rosenblatt, and Irvin S.Y. Chen

We report here the development of a rapid and quantitative method for measuring in vitro T cell transformation by human T cell leukemia viruses type I (HTLV-I) and type II (HTLV-II). This method is based on our finding that cocultivation of lethally irradiated HTLV-producing cells with peripheral blood lymphocytes (PBLs) preactivated for 24 hours with phytohemagglutinin and interleukin-2 (IL-2) induces colony formation in methylcellulose-containing medium. Colonies of about 200 cells can be clearly distinguished from background aggregates within four to six days after cocultivation. These colonies gradually increase

in size and reach 300 to 1,000 cells within 14 days after cocultivation. Cells of these colonies were infected, as evidenced by expression of viral p19 antigen and the presence of HTLV proviral sequences. These cells proved to be transformed in terms of IL-2-independent continuous growth in liquid medium. Colony formation was found to depend in a linear fashion upon the percentage of the infected cells present in the irradiated cell population and is sufficiently sensitive to detect as few as 1% of virus-producing cells.

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THE HUMAN T CELL LEUKEMIA viruses type I (HTLV-I) and type II (HTLV-II) have been etiologically implicated with specific human T cell malignancies.¹ HTLV-I has been established by seroepidemiological and molecular studies as the causative agent of adult T cell leukemia (ATL), an aggressive disease endemic to southern Japan,² the Caribbean basin,^{3,4} Africa,⁵ certain parts of the southeastern United States,⁶ and sporadically in other locations.⁷ HTLV-II has been associated with two cases of a variant of hairy cell leukemia, which, unlike ATL, is a rare and relatively indolent disease.⁸⁻¹¹

Both HTLV-I and HTLV-II are capable of immortalizing normal peripheral blood and umbilical vein T lymphocytes in vitro.¹²⁻¹⁵ Although these viruses can also infect a variety of other cells in culture,¹⁵⁻²¹ their in vitro transforming capacity seems to be restricted to human T cells. The mechanism of cell transformation by HTLV is still unknown and is likely to be different from that of other transforming retroviruses.²² Neither HTLV-I nor HTLV-II carries a classic retroviral oncogene, and there is also no evidence that integration of the proviral DNA occurs in specific sites of the cellular genome in tumors.²³ Clarification of this mechanism would be greatly facilitated if the HTLV-induced transformation of normal T lymphocytes could be measured by a rapid quantitative assay. In previous studies, transformation by HTLV-I and HTLV-II has been determined by cocultivating peripheral or cord blood lymphocytes in liquid cultures with lethally

irradiated or mitomycin-treated HTLV-producing cell lines with or without interleukin-2 (IL-2). Transformation in this manner is defined by the appearance of IL-2-independent immortalized T cells.¹²⁻¹⁵ More recently, it has been found that the efficiency of HTLV-induced transformation can be enhanced by preactivation of the target lymphocytes with phytohemagglutinin (PHA) in the presence of IL-2 for 24 hours before cocultivation with the virus-producing cells.²⁴ However, this procedure is inconvenient because of the length of time required to define IL-2-independent immortalization of the target lymphocytes. Moreover, this assay does not provide a quantitative analysis of the transformation potential.

We have developed a rapid colony assay that can quantitatively determine HTLV-induced transformation within four to six days. This assay relies on our finding that normal lymphocytes, preactivated with PHA in the presence of IL-2, produce colonies after cocultivation with lethally irradiated HTLV-producing cell lines in methylcellulose-containing medium.

MATERIALS AND METHODS

Cells and viruses. A variety of HTLV-I- and HTLV-II-producing T and B cell lines were tested for induction of colony formation by normal lymphocytes. The HTLV-I-producing cells were ME and ET T cell lines isolated from two different ATL patients²⁵; SLB-I, a T cell line generated from peripheral blood lymphocytes (PBLs) of a normal female blood donor by cocultivation with ME cells.²⁶ The HTLV-II-producing cells were Mo-T and NRA T cell lines isolated from the first (Mo)⁹ and the second (NRA)¹¹ variant hairy cell leukemia patients, respectively; JLB-I, a T cell line established from PBLs of the aforementioned blood donor by cocultivation with Mo-T cells¹⁴; Mo-B, an Epstein-Barr virus (EBV)-transformed and HTLV-II-infected B cell line established from the patient Mo¹⁴; J-WIL, an EBV-transformed B cell line (designated WIL) that was infected by cocultivation with Mo-T cells; and 729 pH6neo, an EBV-transformed B cell line (designated 729) that was stably transfected with an infectious cloned genomic DNA of HTLV-II.¹⁸ HUT-78 and MOLT₄ T cell lines²⁷ and 729 and WIL B cell lines were used as HTLV-uninfected controls.

The target normal PBLs were prepared by Ficoll-Hypaque (P.L. Biochemicals, Milwaukee) density centrifugation¹⁴ from fresh blood samples of random male or female blood donors. The banded mononuclear cells were washed twice with Iscove's medium (Irvine Scientific, Santa Ana, CA) containing 20% fetal calf serum (FCS) and antibiotics (Irvine Scientific) and incubated in flasks with this

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medium for two hours at 37°C to remove adherent cells, and the remaining nonadherent cells were used for the colony assay.

Colony formation assay. The virus-producing cells and the nonproducing control cells received fresh Iscove's medium containing 20% FCS two days before the assay. Immediately before plating, their density was adjusted to 10^6 viable cells (determined by trypan blue exclusion) per milliliter and irradiated with 12,000 rad. The target lymphocytes were activated for 24 hours before the assay by 1% PHA in the presence of IL-2. As a source for IL-2, we used a 1:1,000 dilution of conditioned medium collected from cultures of COS cells 48 and 72 hours after transfection with a recombinant eukaryotic expression plasmid carrying the IL-2 gene²⁸ that was provided by S.C. Clark (Genetics Institute, Boston). The conditioned medium contained about 1 ng IL-2/mL. Both the irradiated and target cells were centrifuged and resuspended in Iscove's medium containing 20% FCS by extensive dispersion of cell aggregates through a 25-gauge syringe needle. Aliquots of 10^5 irradiated cells were mixed with an equal number of target lymphocytes in a final volume of 1 mL of Iscove's medium containing 20% FCS and 3.14% methylcellulose (Dow Chemical Co, Midland, MI), and after

repeated dispersion, the mixture was plated in 35-mm-diameter tissue culture dishes with 2-mm grids (Falcon Labware, Oxnard, CA). In some preliminary experiments, IL-2 was also added to this mixture. After one to two hours of incubation at 37°C, the cultures were inspected for background cell aggregates.

Analysis of HTLV antigen expression. Large colonies were picked 14 days after plating, propagated in liquid to about 10^7 cells, and tested for expression of HTLV p19 antigen on their surface as previously described.⁷

RESULTS

Colony formation by HTLV-infected PBLs. When PBLs, preactivated for 24 hours with PHA in the presence of IL-2, were cocultivated with lethally irradiated HTLV-I- or HTLV-II-producing cell lines in methylcellulose, colony formation was consistently observed. In some experiments, IL-2 was included in the methylcellulose plating mixture. Although these conditions allowed the formation of large colonies of transformed cells, they were found to be inconvenient for scoring these colonies because of a heavy background apparently resulting from IL-2-supported proliferation of the nontransformed PBLs. Omission of IL-2 from the plating mixture was subsequently found to permit similar colony formation without significantly affecting their number. More important, elimination of IL-2 arrested the growth of untransformed PBLs and thus reduced the density of the background. Under such conditions, colonies of transformed cells could be easily detected microscopically as early as four to six days after plating. At this early stage, these colonies were relatively small (about 200 cells) but clearly distinguishable from background aggregates of less than 70 cells. Moreover, upon further incubation, the colonies of transformed cells gradually grew in size, whereas the background aggregates remained the same or gradually degenerated. Figure 1A illustrates an example of large colonies, with some small aggregates of apparently dead cells observed 14 days after cocultivating PBLs with HTLV-II-infected Mo-T cells. Such colonies may grow to more than 1,000 cells as shown in Fig 1B. Table 1 presents repeated assays with PBLs from random different normal male and female blood donors that used the same HTLV-producing cell line (Mo-T). Colony formation was consistently detected in all of these experiments, thereby indicating that the assay can be carried out with PBLs of any available healthy blood donor.

Cell morphology in colonies. Large colonies were randomly picked, fixed by cytocentrifugation, and stained with

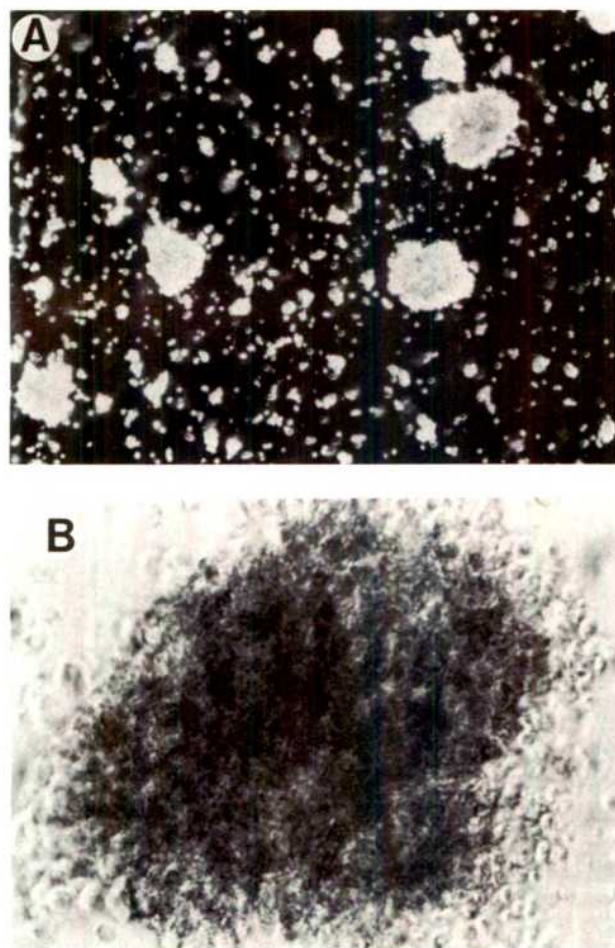


Fig 1. Colonies of PBLs induced by lethally irradiated Mo-T cells. PBLs were activated with PHA (1%) for 24 hours in the presence of IL-2 and then cocultivated with lethally irradiated (12,000 rad) Mo-T cells. (A) Cultures were photographed 14 days after plating. (Original magnification $\times 4$; current magnification $\times 3$.) (B) An individual large colony was photographed at a $20\times$ magnification for a rough estimation of the number of cells in such colonies. This particular colony contains more than 1,000 cells. (Current magnification $\times 15$.)

Table 1. Colony Induction by Mo-T Cell Line in PBLs From Different Blood Donors

Experiment No.	Gender of Blood Donor	No. of Colonies per Dish*		
		PBLs Alone	Mo-T Alone	PBLs + Mo-T
1	F	0	0	512
2	F	0	0	536
3	F	0	0	424
4	F	0	0	560
5	F	0	0	280
6	M	0	0	256
7	M	0	0	648

*Large colonies of more than 300 cells were counted 14 days after plating. Data are presented as averages of duplicate cultures.

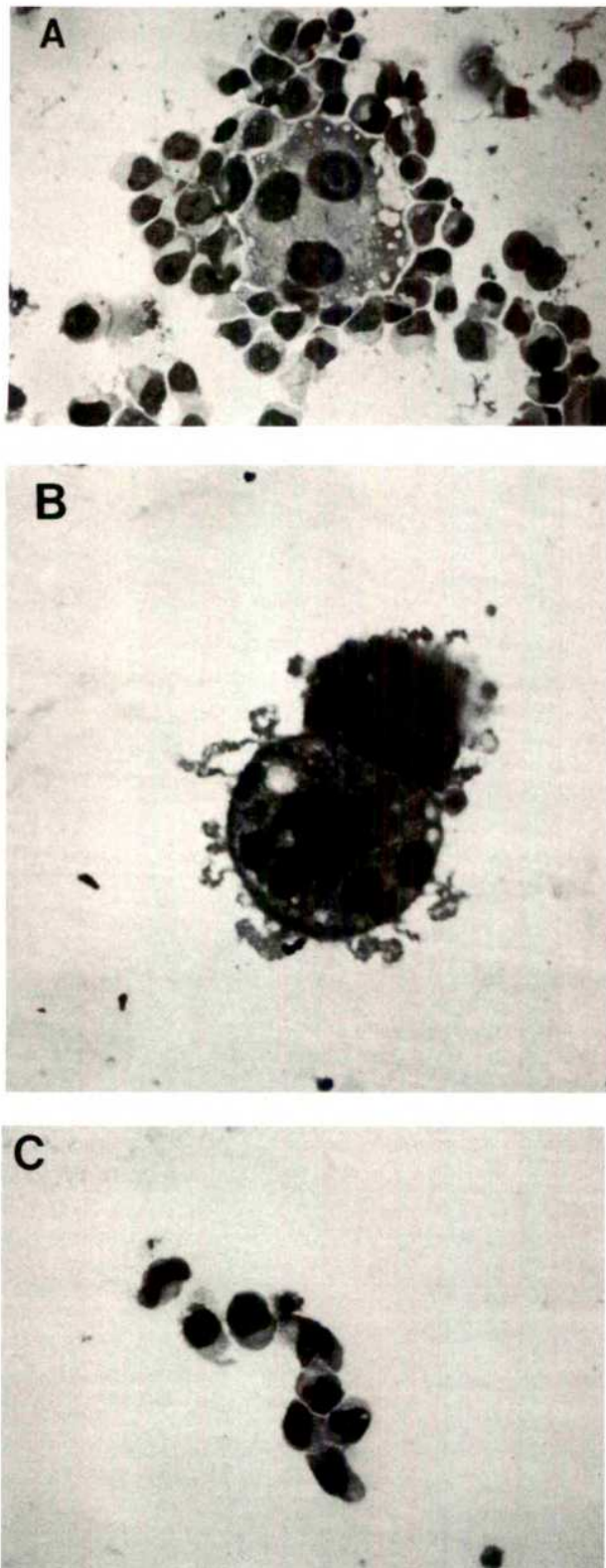


Fig 2. Giemsa staining of colonies. A large colony induced by Mo-T cells (A) (original magnification $\times 25$; current magnification $\times 16$) and a small clump picked from a control culture of irradiated Mo-T cells alone (B) (original magnification $\times 25$; current magnification $\times 25$) or from a culture of PBLs alone (C) were spun down onto a glass slide and photographed (original magnification $\times 25$; current magnification $\times 23$) after Giemsa staining.

Giemsa for morphological analysis. Figure 2A illustrates a representative colony induced by Mo-T cells. It can be seen that the colony consists of mononuclear cells growing around an apparent, degenerating, irradiated virus-producing cell. Such degenerating cells, obtained from clumps picked from dishes containing irradiated Mo-T cells alone, are shown in Fig 2B. Figure 2C, on the other hand, shows mononuclear cells derived from nonspecific aggregates picked from control dishes containing PBLs alone.

Specificity of colony induction by HTLV-producing cells. To verify that this assay was a specific measure for HTLV infection, a variety of HTLV-I- and HTLV-II-producing T or B cell lines was tested in comparison to HTLV-uninfected cells. The data summarized in Table 2 clearly indicate that colony formation was induced, under our experimental conditions, only by the HTLV-producing cell lines, not by any of the tested nonproducing cell lines.

Cell morphology of representative large colonies, randomly picked from each of these experiments for Giemsa staining, was the same as shown in Fig 2A, whereas aggregates picked from mixed cultures of PBLs and uninfected irradiated cells were similar to those shown in Fig 2C for PBLs alone (not shown).

To confirm that the transformed cells were indeed infected by HTLV, we examined the expression of HTLV antigens on the surface of cells from various colonies. Large colonies were randomly picked from various experiments 14 days after plating, grown in liquid medium to about 10^6 cells, and subjected to direct immunofluorescence analysis using monoclonal antibodies against HTLV p19 antigen. A representative experiment is depicted in Table 3. In this particular experiment, colony formation was induced by Mo-T and 729 pH6neo, and as can be seen, all of the tested colonies contained p19-expressing cells, although in variable proportion, ranging from 5% to 95% of their cell population. Similar data were obtained with colonies induced by other HTLV-producing cells (not shown). Such a variation is frequently observed between different HTLV-infected cell clones, apparently reflecting different efficiencies of virus expression in different cells. Final confirmation of HTLV infection

Table 2. Colony-Inducing Capacity of Different HTLV-Infected and -Uninfected Cell Lines

Cells	Cell Lineage	HTLV Type	Colony No. per Dish*
ME	T	I	960
ET	T	I	536
SLB-I	T	I	1,040
Mo-T	T	II	648
NRA	T	II	192
JLB-I	T	II	728
Mo-B	B	II	268
729 pH6neo	B	II	360
J-WIL	B	II	176
HUT-78	T	None	0
MOLT ₄	T	None	0
729	B	None	0
WIL	B	None	0

*Data are presented as averages of duplicate cultures.

Table 3. HTLV Surface Antigen Expression by Colony Cells

Colony-Inducing Cell Line	p19 Antigen Expression (%) [*]
Mo-T	20, 90, 10, 40, 5, 20
729 pH6neo	5, 30, 90, 5, 10, 40

^{*}Several large colonies were randomly picked 14 days after plating, grown in liquid medium to about 10^5 cells, and analyzed by indirect immunofluorescence assay using monoclonal antibodies against HTLV p19 antigen. Data are presented for each individually tested colony in terms of percentage of positive cells.

was made by Southern hybridization (data not shown). We define HTLV cellular transformation as growth in the absence of exogenous IL-2.¹²⁻¹⁵ The transformed state of the cell in individual colonies in our experiments was thus confirmed by their growth in liquid medium without IL-2.

Sensitivity and quantitative application of the colony assay. Finally, we analyzed the sensitivity of the assay by determining the lowest proportion of HTLV-infected cells that it can detect in tested cell populations and whether it is adequate for quantitation of these infected cells. For this purpose, variable numbers of HTLV-infected cells (such as Mo-T and 729 pH6neo) were mixed with uninfected cells (729 cell line) before irradiation, the total cell number of cells in the mixture kept constant. This mixture was cocultivated with activated PBLs. From the results illustrated in Fig 3, it is evident that this assay can detect HTLV-infected cells even if they constitute as few as 1% of the irradiated cell population. Furthermore, the assay is fairly linear within the range of its sensitivity.

DISCUSSION

Both HTLV-I and HTLV-II can infect a variety of cells in culture.^{12,21} They also exhibit an *in vitro* transforming activity, but this activity seems to be restricted to T cells with a mature activated phenotype.^{1,12,13} Our current investigations

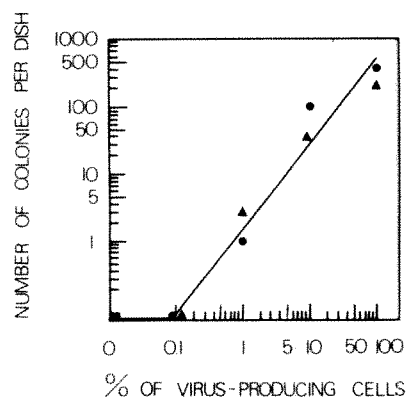


Fig 3. Quantitative correlation between colony formation and the proportion of HTLV-infected cells. Mo-T (filled circles) and 729 pH6neo (filled triangles) cells were mixed with uninfected 729 cells at the indicated proportions, the total number kept at 10^5 cells per culture. These mixtures were lethally irradiated and cocultivated with 10^5 PHA/IL-2 activated target PBLs as detailed in Materials and Methods. Large colonies were counted 14 days after cocultivation.

on the mechanism of the HTLV-induced transformation have motivated us to search for a rapid assay to measure this transforming capacity. Preactivation of target PBLs with PHA and IL-2 before cocultivation with lethally irradiated HTLV-producing cells enhances the formation of IL-2-independent transformed T cells,^{24,29} whereas malignant T cells produce colonies in methylcellulose.³⁰ We therefore elucidated whether preactivated normal PBLs would produce such colonies upon cocultivation with lethally irradiated HTLV-producing cell lines. We examined a variety of T and B cell lines producing either HTLV-I or HTLV-II and showed an efficient and rapid colony formation that could conveniently be used for assaying the HTLV transforming capacity. Such colonies could be visualized and counted as early as four to six days after plating. Cells from such colonies were found to be capable of IL-2-independent continuous proliferation in liquid medium, thus confirming their transformed phenotype.^{1,12,13,24} Furthermore, these cells expressed HTLV p19 antigen, thereby indicating that they were productively infected by HTLV. It is worthwhile to emphasize, in this context, that in contrast to virus expression in such *in vitro* transformed cells, no viral antigen or RNA can be detected in primary leukemic T cells from patients with HTLV-associated T cell malignancies, although these primary cells harbor HTLV DNA in their genomes. Virus expression can be demonstrated in such cells only if they are grown in culture. This contrast may suggest that *in vitro* T cell transformation is probably analogous to an early event that initiates the *in vivo* T cell malignancy by altering the growth regulation of the infected cells. However, in the *in vivo* leukemogenic process, this event is apparently followed by additional events leading to the eventual malignancy that is no longer dependent on viral functions.

Finally, our data demonstrate that the colony assay is sufficiently sensitive to detect HTLV-infected cells, even if they constitute as few as 1% of the examined cell population. Under our experimental conditions, this means that roughly one out of 10^3 virus-producing cells can be detected as an infectious center. Such an infectious center may be derived from more than one PBL that proliferates into a colony; therefore, these colonies are not necessarily monoclonal. However, since the colony-inducing capacity of such populations is linearly proportional to the percentage of the virus-producing cells, this assay can nevertheless be used for quantitative analysis of the HTLV-induced *in vitro* transformation. This quantitative potential of the assay may be useful for examining the early events associated with T cell immortalization by these viruses. It may also provide a tool for correlation between structural and functional properties of various HTLV isolates and mutants.

NOTE ADDED IN PROOF

Since acceptance of this report for publication, another report was published describing a different HTLV colony assay (Graziano et al: Cancer Res 47:2468, 1987).

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Monoclonal Antibodies Against the Chronic Lymphatic Leukemia Antigen cCLLa: Characterization and Reactivity

By Guy B. Faguet and Julia F. Agee

Monoclonal antibodies (MoAbs) were developed against the cCLLa, a 69-kilodalton leukemia-associated antigen expressed on malignant cells of B-type chronic lymphatic leukemia (B-CLL) and its variants: prolymphocytic (PLL) and hairy cell leukemias (HCL). Two hybridomas yielded ~2 and ~7.5 mg/mL of IgG2a κ and IgM κ , respectively. Monoclonal surface immunoglobulin-bearing cells of all B-CLL patients studied ($n = 30$) reacted with the MoAbs ($r > .99$) regardless of stage or lymphocyte count. This suggests that the malignant clone in CLL can be identified and its size monitored by using our MoAbs. In contrast, normal B lymphocytes, a large panel of normal, reactive and neoplastic cells, and malignant cell lines failed to react with either MoAb as judged by indirect immunofluorescence and by flow cytometry. Only two patients (one with non-Hodgkin's lymphoma, the other with acute myeloblastic leukemia)

exhibited a small cell subset reactive with the MoAbs. cCLLa specificity was suggested by selective target cell reactivity and competitive inhibition-absorption and confirmed by immunoprecipitation. MoAbs IgG2a κ and IgM κ appeared to share antigenic determinants and were moderate and avid complement binders inducing 100% and 40% target cell lysis, respectively. cCLLa density on malignant CLL and HCL cells was estimated by equilibrium binding studies using the IgG2a κ MoAb at 1.7 and 9×10^6 /cell, respectively. The restricted expression of the cCLLa and the specificity and cytolytic activity of the anti-cCLLa MoAbs support these antibodies as probes for the classification of lymphoproliferative diseases and for the specific diagnosis and treatment of B-CLL and its variants.

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WE HAVE PREVIOUSLY REPORTED¹ that neoplastic cells from patients with chronic lymphatic leukemia (CLL) of B cell lineage and its variants² prolymphocytic (PLL) and hairy cell (HCL) leukemias express a common surface antigen (cCLLa). This novel gp 69-molecular weight polypeptide appeared unrelated to the HLA complex, the T cell differentiation complex gp 65-71,^{3,5} and the human T cell leukemia virus (HTLV-1).⁶ Immunophenotyping of normal and reactive lymphocytes and of neoplastic cells and cell lines derived from patients with leukemias and lymphomas by using a xenogeneic anti-cCLLa antiserum (α CLL) failed to demonstrate surface cCLLa. Cells and tissues studied included normal circulating and tissue T or B lymphocytes; fetal and cord blood lymphocytes; blood lymphocytes from patients with infectious mononucleosis; circulating, nodal, and splenic lymphocytes from patients with Hodgkin's disease and non-Hodgkin's lymphoma; blast cells from patients with lymphoid and nonlymphoid adult and childhood leukemias; and fetal, B-, and T-derived lymphoblastoid and pluripotent cell lines. This highly selective expression of the cCLLa provides both an ideal marker for diagnosing cCLLa-positive clones in a preclinical and potentially eradicable phase of development and a suitable target for immunotherapy using appropriate monoclonal antibodies (MoAbs).

In this communication we report the development of a library of MoAbs directed against the cCLLa. The specificity, selective reactivity, and characterization of two of the MoAbs (an IgG2a κ and IgM κ) are presented.

MATERIALS AND METHODS

Cells

Normal and reactive blood lymphocytes and circulating blasts obtained from healthy volunteers and patients with infectious mononucleosis and from leukemic patients, respectively, were prepared from heparinized peripheral blood by a modified Ficoll-Hypaque gradient.⁷ CLL patients in this study exhibited clinical disease of B cell lineage with absolute lymphocyte counts $>20,000/\mu\text{L}$. Tissue lymphocytes were teased from fresh biopsy specimens. B- and T-enriched lymphocyte populations were separated according to

published procedures.⁸ The composition and lineage derivation of all cell preparations were assessed by cytomorphology and immunophenotyping before each assay. Myeloid and monocytoid cell contamination was consistently $<3\%$, and cell subtype homogeneity was $>80\%$ and $>90\%$ for enriched B and T lymphocytes, respectively. Lymphoblastoid, fetal, pluripotent, and fibroblastoid cell lines obtained from various sources were cultured in appropriate media and stored in liquid nitrogen. HCL cell lines were established from peripheral blood of two HCL patients according to published procedures.⁹ These cells were shown to be indistinguishable from the HCL cells of origin as judged by cytochemistry, electron microscopy, and immunophenotype, including expression of cCLLa (manuscript in preparation). All cell preparations and manipulations were maintained strictly sterile.

Immunophenotype

Surface immunoglobulins (SIs) were assessed by direct immunofluorescence assay using fluorescein isothiocyanate (FITC) conjugated antisera specific for each heavy and light chains. Other surface determinants were assayed by indirect immunofluorescence assay (IFA) using the α CLL antiserum and the following MoAbs: OKT series (Ortho Diagnostics, Raritan, NJ); Leu series (Becton Dickinson, Mountain View, CA); My4, B1, B2, MO2, and J5 (Coulter Immunology, Hialeah, FL); and the anti-cCLLa MoAbs CLL-1 and CLL-2. Most studies were done by fluorescence microscopy on a Leitz epifluorescence microscope (E. Leitz, Inc, Rockleigh, NJ). Dual fluoroscopy was carried out by flow cytometry (Coulter Electronics, Hialeah, FL) and analyzed by the Quadsat program.

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Monoclonal Anti-cCLLa Antibodies

Mouse anti-cCLLa MoAbs were developed according to the method of Kohler and Milstein.¹⁰ Briefly, female BALB/c mice were immunized by weekly ($\times 2$) intraperitoneal, (IP) injections of 10^7 CLL cells in complete Freund's adjuvant followed by intravenous administration of 50 μ g of purified cCLLa three days before sacrifice. Immune spleen cells were hybridized with Sp2/0 mouse myeloma cells (2:1 ratio) using polyethylene glycol 1000 (Sigma Chemical Co, St Louis). Hybrids were grown (10^5 /well) in 96-well plates containing 5×10^4 nonimmune BALB/c spleen cells as feeder cells. Antibody activity against CLL cells, purified cCLLa, and normal B lymphocytes was ascertained by the enzyme-linked immunosorbent assay technique in 3-week-old supernatants. Antibody-secreting cells were expanded and sequentially cloned twice by limiting dilution technique using peritoneal macrophages as feeder cells. Subcloned hybrids with demonstrated specificity against the cCLLa were implanted IP into BALB/c mice or were aliquoted for storage in liquid nitrogen.

Immunofluorescence and Cytotoxicity Assays

MoAb reactivity against cell surface cCLLa was assessed in cell suspensions by IFA¹¹ using goat antimouse IgG or IgM (Cappel Laboratories, Cochranville, PA) and by antibody-mediated cell lysis as measured by ^{51}Cr -release cytotoxicity.¹² Antibodies were used in concentrations sufficient to achieve saturation binding. A panel of antibodies against B-CLL cells and against purified normal T and B lymphocytes served to characterize these cell subsets and as positive and negative controls.¹ Nonimmune mouse and rabbit antisera and irrelevant antibodies against cCLLa-positive target cells were also included as negative controls.

Complement Binding

Anti-MoAb-mediated CLL cell lysis was assessed by ^{51}Cr release microcytotoxicity assay using human cCLLa and rabbit complement. Briefly, after labeling cells with ^{51}Cr (100 $\mu\text{Ci}/10^6$ cells) for two hours at room temperature, thrice-washed aliquots (5×10^4 cells) were exposed to saturating concentrations of antibody plus complement. After 90 minutes' incubation at room temperature, ^{51}Cr released in the supernatants was counted in a gamma counter (Gamma 4000; Beckman Instruments, Inc, Palo Alto, CA). Negative controls included the use of complement, antibody, or medium alone. The use of a pan-T/B antibody assay with complement served as positive control.

Preparation of cCLLa and of ^3H -cCLLa

CLL membranes were prepared according to a modified published procedure¹³ as previously described.¹ Lyophilized samples from αCLL - or CLL-2-Sepharose affinity column-purified membranes¹⁴ were dissolved in 10 mmol/L phosphate buffer, pH 8.0, and applied to a 10-mL column (0.72×25 cm) of diethyl aminoethyl-cellulose (DE-52, Whatman, Inc, Pierce Chemical Co, Rockford, IL) equilibrated with the same buffer. The protein was eluted with an NaCl gradient to 1.0 mol/L of 0.02 mol/L NaCl. One-milliliter fractions were collected and the protein content ascertained by absorption at 280 nm. Tritiated cCLLa was obtained by passage through an antibody-Sepharose affinity column of membranes derived from cCLLa-positive cells cultured in medium rich in ^3H -LEU.

Gel Electrophoresis and Autoradiography

The molecular weight of native and tritiated cCLLa was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described¹ and by bidimensional gels.

Detection of ^3H -cCLLa in polyacrylamide gels was done by a modified scintillation autoradiographic procedure.¹⁵

Immunoprecipitation of cCLLa

Tritiated cCLLa (1,000 cpm/ μg) was incubated with CLL-2 (1:4 ratio) for two hours at 37°C in 0.5% bovine serum albumin (BSA)-presaturated tubes. A fivefold excess of Staph A protein-Sepharose/Hanks' buffered saline solution (HBSS) in 0.5% BSA was added to each tube and further incubated one hour in a mixer. Tubes were then washed three times with HBSS in 0.5% BSA and centrifuged for one minute at 1,300 g, and the pellets were counted in a scintillation counter (Model LS7500, Beckman Instruments). Controls included irrelevant antigen, irrelevant antibody, and an absence of antibody.

cCLLa Density on Target Cells

The density of cCLLa on target cells and its affinity for the MoAb were assessed from equilibrium binding experiments. Briefly, the IgG fraction of CLL-2 was radioiodinated with reductant-free ^{125}I with a specific activity of 17 Ci/mmol/L (ICN Life Sciences Group, Irvine, CA) by the chloramine-T method.¹⁶ Aliquots of 10^6 target cells suspended in 0.1 mL of minimal essential medium containing 0.1% albumin were incubated in duplicate plastic culture tubes presoaked in phosphate-buffered saline with 0.5% albumin (PBS/albumin), pH 7.2, with ^{125}I -MoAb in concentrations ranging from 1×10^{-3} μg to 10 μg in 0.4 mL of PBS/albumin. After reaching equilibrium the reaction mixture was layered over a 4:1 mixture of dibutyl phthalate and bis-(2-ethylhexyl) phthalate and centrifuged at 10,000 g for two minutes. The cell pellet was removed, and cell-bound radioactivity was measured in a gamma counter. Equilibrium binding data were analyzed by curve fitting using the least-square Ligand program¹⁷ adapted to Apple-Soft Basic for the Apple II+ microcomputer. The goodness of fit of derived parameters was visually judged by graphic analysis^{18,19} as recently proposed.²⁰

RESULTS

Monoclonal Anti-CLL Antibodies

By using the protocol outlined in Materials and Methods, 98 subclones were developed that produced antibodies reacting with CLL cells and with column-purified cCLLa but remained unreactive against normal T and B lymphocytes. Of these, 18 were IgM, and 80 were IgG. Because of their relatively high yield (approximately 5 $\mu\text{g}/\text{mL}$ and 2 $\mu\text{g}/\text{mL}$ of supernatant, respectively) two subclones (an IgM κ and an IgG2 $\alpha\kappa$) were selected for IP implantation in the BALB/c mice. Their hybridomas yielded 7 to 7.5 mg/mL and 1.8 to 2.5 mg/mL of ascitic fluid, respectively. These two MoAbs, designated CLL-1 and CLL-2, respectively, are the focus of the present communication. Most studies reported were carried out with ascitic fluid. However, its purified immunoglobulin (IgG or IgM) content was used whenever appropriate as indicated in the text and legends. All but the cCLLa density studies were conducted at MoAb titers sufficient to provide saturating binding conditions.

Target Specificity of MoAbs CLL-1 and CLL-2

That the cCLLa is the target antigen for both MoAbs was demonstrated as follows.

Reactivity pattern. The first indication is that both the CLL-1 and CLL-2 MoAbs reacted with the cCLLa derived

Table 1. Inhibition of CLL-1 and CLL-2 Binding by Cell Preincubation With α CLL

	CLL Cells			T Cells			B Cells		
	Fresh Preincubated			Fresh Preincubated			Fresh Preincubated		
	RAM	RAM	GAR	RAM	RAM	GAR	RAM	RAM	GAR
CLL-1	85.1	2.9	86.3	1.4	1.4	1.0	3.3	2.9	1.4
CLL-2	83.3	1.4	85.6	1.9	1.9	1.4	2.8	2.5	1.0
OKT-3	8.3	8.1	85.6	93.4	93.0	0.4	6.2	6.2	3.9
LEU-12	87.0	88.0	86.8	3.0	2.5	2.2	89.0	87.0	2.0
LEU-1	88.3	83.9	87.3	92.0	95.3	1.8	3.3	1.4	1.0
α CLL	2.5	1.7	85.6	0.5	0.5	0.5	2.3	2.3	1.8

Aliquots of CLL, T, or B cells (10^6 /well) in RPMI 1640 preincubated or not with α CLL (20 μ L/well) were further incubated (60 minutes at 22°C) with 5 μ g of the antibodies listed (except α CLL, 10 μ L). After washing three times, reactive cells were assayed by IFA using FITC-conjugated rabbit antimouse (RAM) antibodies (except goat antirabbit (GAR) antibodies for detection of α CLL). All assays were done in triplicate. Results are expressed as the percentage of reactive cells.

from the initial screening of hybrids, which involves assaying all hybridized clones and subclones for antibody production against cCLLa-positive and negative target cells (preselected by phenotyping with the xenogeneic anti-cCLLa antibody α CLL¹ and against purified cCLLa). Additionally, paired analysis of the cCLLa-specific α CLL reactivity v that of either MoAb showed a correlation coefficient of >.99.

Competitive inhibition of CLL-1 and CLL-2 binding by α CLL. CLL cells and normal T and B lymphocytes were incubated with or without α CLL before exposure to T-, B- and cCLLa-specific MoAbs, respectively. The reactivity of CLL-1 and CLL-2 with fresh CLL cells (85% and 83%, respectively) was completely abrogated (to nonspecific background levels) by α CLL preincubation (Table 1). In contrast, the expected reactivity of MoAbs OKT3 with T lymphocytes (93%), that of LEU-12 with CLL cells and normal B lymphocytes (87% and 89%, respectively), and that of LEU-1 with T lymphocytes and CLL cells (88% and 92%, respectively) was unaffected by preincubation of target cells with α CLL. α CLL-preincubated CLL cells reacted with fluoresceinated goat antirabbit antibody, thereby confirming cCLLa occupancy by α CLL and therefore the specificity of the competitive inhibition.

Competitive inhibition of CLL-2 binding to cCLLa-positive target cells by purified cCLLa. The reactivity of CLL-2 was assayed against CLL and HCL cells by IFA before and after absorption with purified cCLLa derived from α CLL-immunoabsorbent columns. As shown in Table 2, although the CLL-2 mediated the fluorescence of 92% of CLL and 51% of HCL target cells assayed, a complete abrogation (to nonspecific background levels) of these reactions was observed when using cCLLa-absorbed CLL-2. In contrast, preabsorption of the irrelevant antibody LEU-1 with purified cCLLa had no effect on its reactivity with CLL cells (95%) or with normal T lymphocytes (89%). T lymphocytes were included as cCLLa-negative, LEU-1-positive control target cells.

Immunoprecipitation of cCLLa by CLL-2. Tritiated cCLLa was obtained by passage through α CLL-Sepharose affinity chromatography of membranes derived from

Table 2. Inhibition of CLL-2 Binding by cCLLa

Reagents	CLL Cells	HCL Cells	T Cells
CLL-2			
Unabsorbed	92.3	51.4	2.2
cCLLa absorbed	2.9	3.6	1.4
Inhibition	96.4	91.4	0.0
LEU-1			
Unabsorbed	95.4	3.3	89.2
cCLLa absorbed	95.2	3.7	97.7
Inhibition	0.0	0.0	0.0

Cell aliquots (10^5 /well) were incubated for 60 minutes at 22°C with CLL-2 (10 μ g/well) in RPMI 1640 before and after absorption with column-purified cCLLa (10:1 wt:wt ratio). After washing three times, FITC-conjugated rabbit antimouse IgG, 10 μ g/well, was added and incubated for 60 minutes at 22°C, and reactive cells were assessed by IFA. CLL and HCL cells were used as cCLLa-positive target cells. MoAb LEU-1 and cCLLa-negative T cells were used as controls. All assays were done in triplicate. Results are expressed as the percentage of reactive cells or as percent inhibition.

cCLLa-positive cells cultured in medium rich in ³H-LEU. HCL lines were chosen because these cCLLa-positive cells exhibit higher metabolic rates and a shorter replication time than CLL cells (manuscript in preparation). The ³H-cCLLa so obtained demonstrated similar electrophoretic mobility to α CLL-derived cCLLa¹ as shown by SDS-PAGE autoradiography (Fig 1). The ³H-cCLLa completely abrogated the CLL-2 reactivity with CLL target cells and was immunoprecipitated by CLL-2 but not by irrelevant antibody or Sepharose alone, nor were irrelevant polypeptides precipitable by the CLL-2 (not shown).

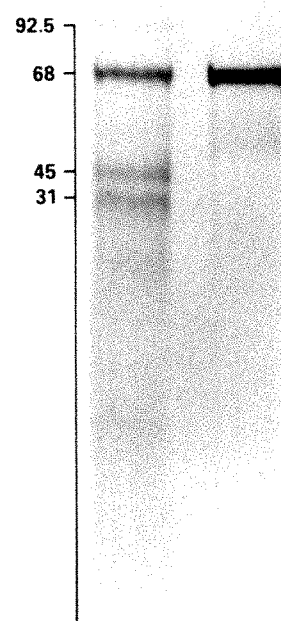


Fig 1. Autoradiography of SDS-PAGE slab gel showing the ³H-cCLLa on the right lane compared with ¹²⁵I-labeled molecular weight markers on the left lane (phosphorylase β , 92.5 kilodaltons [kd]; BSA, 68 kd; ovalbumin, 45 kd; and carbonic anhydrase, 31 kd).

Table 3. Competitive Cross-Inhibition of CLL-1 and CLL-2 Binding

Reagents	Second Antibody		
	None	CLL-1	CLL-2
CLL cells + CLL-1	96.0	—	3.6
CLL cells + CLL-2	96.0	3.4	—
Inhibition	—	96.5	96.3

Cell aliquots (10^5 /well) were incubated with supersaturating concentrations of CLL-1 or CLL-2 (100 μ g/well) for two hours at 22°C in RPMI 1640. After washing three times, cells were further incubated for one hour at 22°C with CLL-2 or CLL-1 (10 μ g/well), respectively. Cells reacting with the latter antibodies were assessed by IFA using FITC-conjugated rabbit antimouse IgG or IgM, respectively. All assays are done in triplicate. Results are reported as the percentage of reactive cells or as percent inhibition.

Antigenic Determinants of MoAbs CLL-1 and CLL-2

To assess whether CLL-1 and CLL-2 share antigenic determinant, the following experiments were conducted: CLL cells were first incubated two hours at 37°C with CLL-1 or CLL-2 at concentrations 1,000-fold greater than that needed to saturate all cCLLa binding sites. Cells were then washed three times and examined for reactivity with the CLL-2 or CLL-1, respectively, by IFA. As shown in Table 3, the reactivity with either antibody was completely abrogated by presaturation with the alternate antibody.

Complement Binding of MoAbs CLL-1 and CLL-2

Complement (C) binding of MoAbs CLL-1 using human or rabbit C was assessed by a microcytotoxicity assay on target CLL cells obtained from patients at different disease stages. Table 4 shows results from four representative

patients whose cCLLa-positive cell subsets accounted for 22%, 35%, 74%, and 90% of their respective circulating lymphocytes. CLL-2 bound human C most effectively in that 21.9%, 41.4%, 75.0%, and 92.7% of the target cells were lysed, respectively. Conversely, CLL-1-dependent human C-mediated lysis was 7.7%, 23.2%, 29.5%, and 41.4%, respectively. Virtually identical results were obtained with rabbit C. When expressed as a function of cCLLa-positive cells within the target cell population assayed, it becomes clear that CLL-2 was 100% lytic to cCLLa-positive cells whereas CLL-1 induced approximately 40% lysis. Antibodies alone showed no lysis above background. As expected, the pan-T/B lymphocyte MoAb (used as positive control) lysed virtually all lymphocytes (88.5% to 93%) with one single exception (62.9%). In each case cytolysis was calculated as the percentage of total lysis induced by Triton X-100.

cCLLa density on CLL and HCL cells. It was calculated that both CLL and HCL cells exhibit a small fraction (70×10^3 and 300×10^3 /cell, respectively) of high-affinity (1×10^9 mol/L⁻¹) MoAb binding sites, and a large component (1.7×10^6 and 9×10^6 /cell, respectively) of lower-affinity sites (1×10^7 mol/L⁻¹). The goodness of fit of the estimated parameters is displayed in Scatchard¹⁸ and semi-logarithmic¹⁹ coordinates (Fig 2). The interpretation of our binding data as indicating two classes of independent sites rather than one class of interacting binding sites on target cells derives from our recent demonstration²¹ that curvilinear Scatchard plots such as these reflect heterogeneous classes of sites with different and fixed affinity. When calculated as a function of cell surface area, the cCLLa density on CLL and HCL cells was equivalent.

Table 4. Complement Binding of MoAbs CLL-1 and CLL-2

Reagents	Patient 1		Patient 2		Patient 3		Patient 4	
	cpm	Lysis (%)	cpm	Lysis (%)	cpm	Lysis (%)	cpm	Lysis (%)
None	248	6.5	1,015	8.9	335	3.6	308	3.3
Human C	183	4.8	847	7.4	244	2.6	407	4.3
Rabbit C	ND	ND	ND	ND	364	3.9	354	3.8
Triton X-100	3,790	100.0	11,392	100.0	9,279	100.0	9,401	100.0
Pan-T/B Antibody*								
Alone	284	7.5	1,035	9.1	380	4.1	350	3.7
+ Human C	3,473	91.6	7,165	62.9	8,215	88.5	8,736	92.9
+ Rabbit C	ND	ND	ND	ND	8,265	89.1	8,744	93.0
CLL-1:								
Alone	265	7.0	1,013	8.9	393	4.2	410	4.4
+ Human C	293	7.7	2,646	23.2	2,737	29.5	3,742	39.8
+ Rabbit C	ND	ND	ND	ND	2,813	30.3	3,680	39.1
CLL-2								
Alone	207	5.5	918	8.1	363	3.9	365	3.9
+ Human C	831	21.9	4,712	41.4	6,958	75.0	8,717	92.7
+ Rabbit C	ND	ND	ND	ND	7,013	75.6	8,470	90.1

Abbreviation: ND, not determined.

Lymphocytes were obtained from four CLL patients whose cCLLa-positive cell subsets represented 22% (1), 35% (2), 74% (3), and 90% (4) of their respective total lymphocyte count. Cells from each patient were incubated in RPMI 1640 with ⁵¹Cr (100 μ Ci/ 10^7 cells) for two hours at 37°C. After washing three times, cell aliquots (5×10^4 /well) were exposed to pan-T/B, CLL-1 or CLL-2 antibody (5 μ g/well) plus human or rabbit C (10 μ L/well), to each reagent alone, or to media alone. After 90 minutes' incubation at 37°C, supernatants were counted in a Beckman gamma counter. All assays were done in triplicate. Results are expressed as supernatant counts (cpm) and as cell lysis (percentage of Triton X-100-induced total lysis).

*Pan-T/B antibody refers to a C-binding MoAb reactive against both T and B lymphocytes.

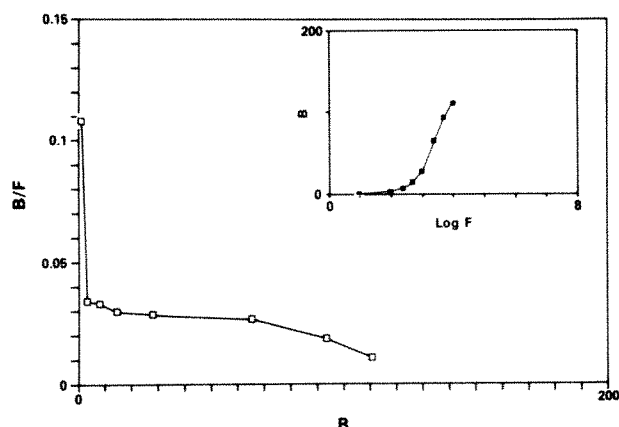


Fig 2. Scatchard and semilogarithmic (inset) plot of ^{125}I -CLL-2 (IgG2a) binding to cCLLa on cells from patients with advanced disease ($n = 2$).

Reactivity of the MoAbs CLL-1 and CLL-2

MoAb CLL-1 reacted with 43% to 97% (mean, 84%; SEM, 6%) of blood lymphocytes from all cases ($n = 32$) of B-CLL in this study (Table 5). Likewise, 45% to 98% (mean, 85%; SEM, 6%) bore monoclonal SIgs. In each case, numbers of circulating cells expressing cCLLa and monoclonal SIgs was directly proportional to the extent of disease as judged by degree of lymphocytosis (Fig 3) and to a lesser extent by clinical stage (not shown). In addition, the absolute or relative numbers of cells expressing cCLLa and monoclonal SIgs correlated highly ($r > .99$) in all cases (Fig 3, inset). Patients with early-stage B-CLL and absolute lymphocyte counts $<20,000/\mu\text{L}$ ($n = 33$) exhibited correspondingly fewer monoclonal SIg- and cCLLa-positive cells. However, good correlations were also observed between monoclonal SIg- and cCLLa-positive cell subsets and between each subset and extent of disease. It was clear that, as CLL progressed from incipient to advanced disease,²² cells bearing monoclonal SIgs and cCLLa accumulate and become the predominant blood lymphocytes. Identical observations were made when using MoAb CLL-2 on the same patient populations (not shown). The intensity of the cCLLa reaction with CLL-2, shown in Fig 4, is compared with that of the B cell differentiation antigen LEU-12. SeD, a cell line derived from a patient with CLL²³ was also reactive. Likewise, cells from five patients with HCL of B lineage also expressed the cCLLa (57%; SEM, 5%) as did cell lines EH and HK derived from two such patients. Three patients with the rare T suppressor (89% to 98% OKT8-positive cells) CLL²⁴ were nonreactive, as was one patient with HCL and one with Sézary syndrome, both with T helper phenotype (72% to 75% OKT4-positive cells). The smaller fraction of cCLLa-positive cells in blood of HCL patients and in cell lines compared with that of patients with advanced CLL possibly relates to circulating clone size and cell cycle-dependent cCLLa expression, respectively. In contrast, $<4\%$ of PBL, T cells (97% OKT3-positive), and B cells (85% SIg-positive) obtained from normal donors reacted with CLL-1 or CLL-2. This reactivity was shown by flow cytometry to represent nonspecific background rather than a small

Table 5. Reactivity of MoAb CLL-1

Target Cells	Tests (n)		Reactive Cells*
	Total	Positive	
Cells bearing the cCLLa			
CLL (B phenotype)	32	32	84 (6)
CLL cell line (SeD)	1	1	37 (4)
PLL (B phenotype)	4	4	85 (6)
HCL (B phenotype)	5	5	57 (5)
HCL cell lines (EH & HK)	2	2	55 (4)
Cells not bearing the cCLLa			
Normal lymphoid cells	45		
PBL lymphocytes	11	0	0-4
T cells (97% [SEM, 4] OKT3 +)	11	0	0-4
B cells (85% [SEM, 4] SIg +)	11	0	0-4
Infectious mononucleosis	12	0	0-4
Malignant cells	63		
Leukemias	26	0	0-4
Acute leukemias			
ALL (adulthood)	5	0	0-4
AML	14	1†	0-4
AMMoL	2	0	0-4
APrML	2	0	0-4
CML in blastic crisis	3	0	0-4
Lymphomas	19	0	0-4
Non-Hodgkin's lymphoma			
PBL	12	1†	0-4
Lymph node cells	5	0	0-4
Hodgkin's disease			
Lymph node cells	1	0	0-4
Splenocytes	1	0	0-4
Cell Lines	18	0	0-4
Lymphoblastoid			
Fetal cell lines	1	0	0-4
B cell lines	7	0	0-4
T cell lines	4	0	0-4
Pluripotent cell lines	2	0	0-4
Fibroblast cell lines	4	0	0-4

Reactivity of MoAb CLL-1 against normal and malignant cells was assayed by both ^{51}Cr release microcytotoxicity and IFA and showed comparable results. Only immunofluorescence data are shown here.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; AMMoL, acute myelomonoblastic leukemia; APrML, acute promyelocytic leukemia; CML, chronic myelocytic leukemia; PBL, peripheral blood lymphocytes.

*Reactive cells are reported as the percentage (SEM) of 600 cells minimum per assay. Negative controls are reported as main.

†One patient with AML and one with lymphoma exhibited 29% and 45% cCLLa-positive cells in circulation, respectively. All others were negative ($<4\%$).

subset of cCLLa-positive normal cells. None of the patients with ALL, AMMoL, APrML, or CMLBC expressed the cCLLa. However, one of 14 AML patients tested exhibited a small population (29%) of cCLLa-positive cells. Blasts from that patient seemed arrested at a pluripotent stem cell phase of development as suggested by their reacting with T- (31% OKT3, 33% LEU-1), B- (26% SIgM, 24% LEU-12), myeloid- (29% LEU-M4), monocytoid- (34% My4), and HTLV-1 (18%)-specific antibodies. Of 19 Hodgkin's and non-Hodgkin's lymphoma patients examined to date, one

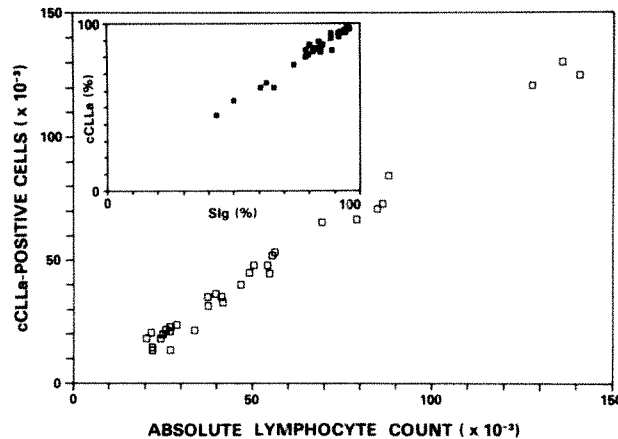


Fig 3. Correlation between lymphocyte counts and cCLLa-expressing cells in blood of CLL patients ($n = 32$) with lymphocyte counts $>20,000/\mu\text{L}$ of blood. Inset shows the correlation between monoclonal SIg- and cCLLa-bearing cells in the same patient population.

individual with diffuse well-differentiated lymphoma was found to exhibit a subpopulation (45%) of cCLLa-positive circulating cells. Finally, none of the 18 cell lines of lymphoid, fetal, myeloid, fibroblastic, and pluripotent cell derivations studied to date expressed the cCLLa.

DISCUSSION

In this communication we report the generation of MoAbs with high specificity against the cCLLa, a recently described

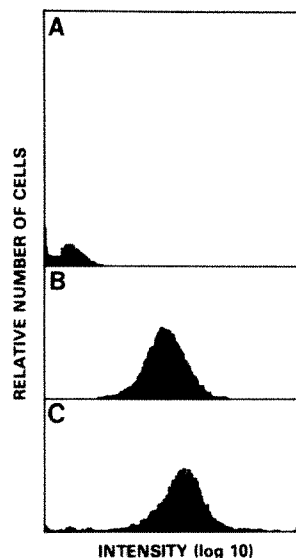


Fig 4. Histogram of indirect immunofluorescence-stained circulating lymphocytes from a CLL patient with 47,000 lymphocytes/ μL . Aliquots of 0.25×10^6 cells were incubated with 5 μL of either normal mouse serum, CLL-2, or LEU-12 for 60 minutes at 22°C. After washing twice, cells were further incubated with saturating concentrations of the FITC-conjugated second antibody for 60 minutes at 22°C. Results are expressed as the relative cell distribution according to fluorescence intensity for cells expressing the cCLLa (B) or LEU-12 (C) compared with the negative control (A).

surface antigen common to neoplastic cells of patients with CLL, PLL, and HCL of B lineage but absent on normal T or B lymphocytes and other normal and neoplastic cells.¹ cCLLa target specificity of MoAbs CLL-1 and CLL-2 was suggested by (a) IFA studies using membrane- or plate-bound antigen and (b) competitive inhibition of the antigen-antibody reaction by either antibody absorption with purified cCLLa or by antigen absorption with the alternate MoAb and was confirmed by immunoprecipitation of the antigen. HCL cells used as the source of both membrane-bound antigen and of purified antigen confirmed that the cCLLa is indeed shared by CLL and HCL cells of B cell lineage as previously suggested.¹ In contrast to the universality of cCLLa expression by circulating neoplastic CLL cells regardless of clone size, not all cells within HCL and CLL lines were positive for this antigen. This might indicate clonal heterogeneity or the impact of the cell cycle on cCLLa expression. The reactivity of our MoAbs ascertained against large panels of normal and neoplastic cells of a variety of sources confirmed the reported restriction of cCLLa expression to the malignant cells from patients with CLL and its variants.¹ The only two exceptions include a patient with undifferentiated AML whose blasts expressed multiple phenotypes including the cCLLa (29%) and one with diffuse well-differentiated lymphoma with cCLLa-positive cells (45%) in circulation. The former observation, not entirely unexpected given the common origin of B lymphocytes and myeloid cells,²⁵ suggests that the cCLLa might represent a differentiation antigen transiently expressed at a very early phase of normal cell development. Our data suggest that, although CLL, PLL, and HCL share a common origin in a cCLLa-positive stem cell, other leukemias rarely originate from that cell at that stage of differentiation. The small number of lymphoma patients studied to date precludes estimating whether cCLLa-positive lymphoma represents a distinct subset of patients with the disease or whether such cases are merely a clinical variant of CLL. The latter appears likely by virtue of the clinical, biologic, histological, and phenotypic similarities between at least certain histological types of lymphomas and CLL.

In the present and other studies,²⁶ the relative size of the circulating cCLLa-positive cell subset in CLL correlated highly with that expressing monoclonal SIgs regardless of stage or degree of lymphocytosis. This suggestion that monoclonal cells express concomitantly SIgs and the cCLLa were amply confirmed by flow cytometry studies of dual-labeled cells (not shown). These observations, the restricted cCLLa expression in B-CLL and lineage-related disorders, and the target specificity of our anti-cCLLa MoAbs suggest their potential for the immunophenotypic classification of leukemias and lymphomas²⁷ and for the objective diagnosis of CLL or its variants regardless of stage. Indeed, by using the cCLLa as a marker, the immunophenotypic diagnosis of CLL is now feasible even in patients with marginal clinical disease because such patients exhibit a heretofore recognizable neoplastic clone in circulation.²⁶ Thus, detection of cCLLa-positive cells in patients with lymphoproliferations of dubious clinical significance is presumptive evidence of the neoplastic nature of these cells and of a common origin and

perhaps etiology for diseases expressing this antigen. Finally, the high cLLa density on the membrane of positive cells and the high cytolytic activity of anti-cLLa MoAbs such as the CLL-2 suggest their immunotherapeutic potential. This hypothesis is supported by ongoing studies in our laboratories that have shown that a single infusion of CLL-2 in nude mice bearing cLLa-positive tumor implants retards tumor growth and doubles the survival of the hosts (manuscript in

preparation). Data presented in this report support the view that cLLa-specific MoAbs are highly sensitive probes to identify cLLa-positive clones at any stage of clinical development and suggest their therapeutic potential.

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Intermediate- to High-Grade Histology of Lymphomas Carrying t(14;18) Is Associated With Additional Nonrandom Chromosome Changes

By Mark E. Richardson, Chen Quanguang, Daniel A. Filippa, Kenneth Offit, Alice Hampton, Prasad R.K. Koduru, Suresh C. Jhanwar, Philip H. Lieberman, Bayard D. Clarkson, and R.S.K. Chaganti

We describe additional nonrandom chromosome abnormalities in 18 cases of intermediate- to high-grade non-Hodgkin's lymphoma (NHL) bearing t(14;18) that were ascertained in a prospective cytogenetic study of all lymphomas seen at Memorial Hospital during the period January 1, 1984, to December 31, 1986. These included seven cases that had histological evidence of transformation from a lower grade and 11 that lacked such evidence. The most common of the additional changes seen in both groups affected chromosomes 6 and 7 and comprised the loss of

chromosome 6 or del(6q) and the presence of more than two copies of chromosome 7 or duplication of 7q. Changes affecting these two chromosomes were less frequent in low-grade lymphomas with t(14;18) as well as in lymphomas lacking the translocation. These data suggest that common cytogenetic mechanisms underlie expression of high-grade histologies by lymphomas carrying t(14;18). In addition, they may serve as indicators of transformation when encountered in low-grade lymphomas with t(14;18). ©1987 by Grune & Stratton, Inc.

RECENT COMBINED HISTOLOGICAL and cytogenetic studies of non-Hodgkin's lymphoma (NHL) have revealed a close association between certain histological patterns and specific reciprocal translocations.¹⁻⁶ Notable among these is the association between follicular low-grade NHL and t(14;18)(q32;q21). In fact, this is the only commonly recurring abnormality among low-grade lymphomas, which suggests that it is of etiologic significance in these lesions.¹⁻⁶ Such a view is further supported by the recent observation that in these translocations *bcl-2*, the gene located at 18q21, undergoes deregulation as a result of its transposition into the immunoglobulin heavy chain (IgH) locus at 14q32.^{7,8} However, some cases of intermediate- to high-grade NHL also have been shown to display t(14;18), usually as part of complex karyotypic changes. Such cases often have exhibited histological evidence of transformation from low-grade follicular lesions.⁶ Cytogenetic studies previously have identified 11 cases of low-grade NHL that transformed to intermediate or high-grade and yet retained t(14;18).^{1-4,8-10} The status of recurrent chromosome abnormalities in addition to t(14;18) associated with such grade transformation has previously not been addressed. Here we report cytogenetic analysis of 18 intermediate- to high-grade NHL with t(14;18), seven of which have had documented evidence of transformation from a lower to a higher grade and 11 have lacked such evidence. Highly nonrandom changes involving chromosomes 6 and 7 were noted in both groups, which suggests that genetic mechanisms mediated by

these changes underlie expression of higher-grade phenotypes by lymphomas with t(14;18). In addition, they may serve as indicators of lymphoma transformation and patient prognosis when encountered in low-grade lymphomas with t(14;18).

MATERIALS AND METHODS

We have been performing prospective cytogenetic, histological, cell marker, and molecular studies of all lymphoma specimens submitted to the pathology department of Memorial Hospital for diagnostic evaluation since January 1, 1984. Correlation studies involving histological, cytogenetic, and cell marker phenotypes of the tumors ascertained through April 1984 have been published elsewhere.⁶ The present report is based on all tumors ascertained between January 1984 and December 1986. This series comprised 168 specimens with a confirmed diagnosis of NHL removed from 167 patients and included lymph node, spleen, and nonlymphoid tissues (fibrous tissue, muscle, lung, testis, etc). All tissue samples were divided, with portions submitted for cytogenetic and molecular genetic studies. The former were processed for chromosome analysis following previously described methods.⁶ The latter was snap frozen in liquid nitrogen and stored at -70°C until studied. Portions destined for histology were fixed in B3, and slides were prepared and stained with hematoxylin and eosin by conventional methods. NHL specimens were retrospectively classified according to the working formulation.¹¹ Bone marrow, pleural effusion, and ascites tumors, although studied, have been excluded from this analysis because they can not be classified by the working formulation. All tissues used in this study were submitted to the laboratory for diagnostic evaluation; informed consent for diagnostic evaluation was obtained at the time of admission to Memorial Hospital.

RESULTS

From this series of lymphomas, clonal chromosome abnormalities were identified in 107 cases; the remaining either showed normal karyotypes only or were cytogenetic failures. One clonally abnormal case was excluded from the analysis because poor chromosome morphology did not permit analysis beyond detection of a t(14;18). Of the 106 remaining cases, 35 showed t(14;18), 17 of which were classified as low grade (12 small cleaved cell follicular and five mixed follicular) and 18 as intermediate to high-grade tumors. Of the latter 18, seven had evidence of histological transformation from a previous lower grade as documented by evaluation of initial and rebiopsy slides (designated here as group I lesions), and 11 lacked such evidence (designated here as

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group II lesions). Recurrent chromosome changes in addition to t(14;18) found in the 18 groups I and II patients were compared with those seen in the 17 low-grade tumors with t(14;18) as well as with those seen in the remaining 71 clonally abnormal NHL lacking t(14;18).

Data on recurrent chromosome changes, cell surface markers, histology, and clinical features of patients belonging to groups I and II are summarized in Table 1. All seven group I patients had received chemotherapy before the

rebiopsy sample that showed transformation; of these, three died within 1 month after transformation, three have not gone into remission after 4 to 25 months of treatment, and one patient is in complete remission 15 months after transformation. In six of the seven cases, cytogenetic studies were obtained after transformation had occurred (Table 1). Three of the eleven group II patients had received treatment at the time of cytogenetic analysis; four died within 4 months of diagnosis, five are alive under treatment for recurrent disease

Table 1. Karyotypic, Immunologic, Clinical, and Histological Features of Intermediate- to High-Grade NHL With t(14;18)

Patient No.	Age/Sex	Site	Surface Markers	Recurrent Chromosome Abnormalities*	Initial Biopsy Histology	Date	Repeat Biopsy Histology	Date	Survival† (mo)	Status	Treatment
Group 1 (tumors with histological evidence of transformation)											
1	68/M	LN	ND	ND	SCC-F	1/81					CYT/Prednisone
	71	ST	IgM λ	+ 7,t(14;18)			SCC-D	11/84	25 +	PR	M2, CYT/ADR
2	54/F	LN	ND	ND	SCC-F	4/79					CYT/Prednisone
	59	LN	IgMA	+ 2, + 5, + 19, + 20, + 21,t(14;18)			LCC-D	8/84	1/2	Exp	BLEO
3	40/M	ST	ND	ND	SCC-F	10/67					RT
	57	LN	IgM λ	ND‡			SCC-D	5/84			NHL-4
	59	LN	ND	+ 5, + 19, + 20, del(6q), t(14;18)			LCC-D&F	8/86	4 +	PR	MACOP-B
4	38/F	SC	ND	ND	Mix-F&D	5/67					RT, Chlorambucil
	55	LN	IgG κ	t(14;18)			SCC-F	2/84			NHL-4
	56	LN	IgM κ	+ 21,t(14;18)			Imb-D	9/85	15 +	CR	M-BACOD
5	61/F	LN	ND	ND	SCC-F	12/79					RT, Chlorambucil
	67	ST	INC	- 3, - 6, - 9, + 7, + 12, del(6q), t(14;18)			LCC-D	4/85	20 +	PR	CHOP
6	57/F	LN	ND	ND	LC-D	9/84					CHOP/BLEO
	58	LN	IgGA	- 1, + 7, + 8, t(14;18)			Imb-D	3/85	1	EXP	MTX/VP 16
7	40/M	LN	ND	ND	SCC-F	2/67					CYT/Prednisone
	54	LN	IgM λ	- 1, - 3, del(6q), t(14;18)			SCC-D	8/84	4	EXP	CHOP
Group 2 (tumors without histological evidence of transformation)											
8	63/M	LN	IgG κ	- 9, + 2, + 5, + 7, + 18, t(14;18)	LNCC-D	6/84			1	EXP	M2, CVP
9	70/F	ST	IgG κ	+ 5, + 7, + 12, t(14;18)	LNCC-D	4/85			19 +	CR	M2
10	68/M	ST	IgM κ	- 1, - 3, - 9, dup(11q), t(14;18)	LNCC-D	2/85			3	EXP, PR	C-MOPP
11	74/F	LN	IgM κ	- 6, + 5, + 7, t(14;18)	LCC-F	9/84			26 +	PR	CHOP
12	45/F	ST	INC	+ 7, t(14;18)	Mx-D	2/86			10 +	PR	MACOP-B
13	84/F	ST	ND	+ 7, + 12, t(14;18)	LCC-D	3/86			4	EXP, PR	CHOP
14	51/F	ST	IgG κ	+ 7, t(14;18)	Imb-D	8/86			4	EXP	RT, MACOP-B
15	53/M	LN	IgM λ	ND	LCC-D	1/85				PR	CHOP, BLEO
	54	LN	ND	ND			LCC-N	5/86			
	54	LN	ND	- 6, + 7, dup(11q), t(14;18), + der(18)			LCC-D	12/86	24 +	PR	L-20
16	72/M	LN	ND	ND	Imb-D	8/83				CR	CHOP
	75	LN	IgM λ	+ 12, t(14;18), + der(14), + der(18)			Imb-D	11/86	41 +	PR	10 EDAM
17	73/F	LN	IgM κ	ND	Imb-D	12/85				PR	MACOP-B
	74	LN	IgM κ	dup(7q), t(14;18), + der(14)			Imb-D	12/86	12 +	PR	MACOP-B
18	46/F	LN	IgG κ	t(14;18)	LCC-F	1/86			12 +	CR	Prednisone
											Chlorambucil

Abbreviations: LN, lymph node; ST, soft tissue (fibrous, adipose, muscular); ND, not determined; INC, inconclusive; SCC, small cleaved cell; SNCC, small noncleaved cell; LC, large cell; LCC, large cleaved cell; LNCC, large noncleaved cell; Mx, mixed small and large cell; Imb, immunoblastic; F, follicular; D, diffuse; EXP, expired; PR, partial response; CR, complete response; M2, vincristine, cyclophosphamide, BCNU, prednisone, melphalan; MACOP-B/M-BACOD, methotrexate, doxorubicin, cyclophosphamide, vincristine, dexamethasone/prednisone, bleomycin; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; CVP, cyclophosphamide, vincristine, prednisone; C-MOPP, cyclophosphamide, vincristine, procarbazine, prednisone; 1-20, vincristine, cyclophosphamide, doxorubicin, methotrexate, daunorubicin, ara-C, L-asparaginase, 5MP, prednisone, BCNU; NHL-4, thiotepa, vincristine, chlorambucil, prednisone; EDAM, deazaoaminopterin; RT, radiation therapy; CYT, cyclophosphamide; ADR, doxorubicin; BLEO, bleomycin; MTX, methotrexate.

*Nonrecurrent chromosome abnormalities are not included. Detailed karyotypes may be obtained from the authors (R.S.K.C.).

†Survival from time of final tissue diagnosis.

‡A bone marrow sample of July 16, 1984, revealed the recurrent abnormalities del(6q), t(14;18).

10 to 41 months after diagnosis, and two are alive in complete remission after chemotherapy 12 to 19 months after diagnosis (Table 1).

Recurrent chromosome abnormalities in addition to t(14;18) were identified in both groups, and those affecting chromosomes 6 and 7 were the most frequent among them. Loss of chromosome 6 or del(6q) was seen in four of these 18 cases (22.2%) (nos. 3 and 5 of group I and 11 and 15 of group II). Similar abnormalities affecting chromosome 6 were seen in three of 17 low-grade NHL (17.7%) with t(14;18) and nine of 71 (12.76%) NHL without t(14;18) in our series. More than two copies of chromosome 7 or duplication of 7q were identified in 12 of the 18 (66.7%) cases (nos. 1, 5, and 6 of group I and 8, 9, 11, 12, 13, 14, 15 and 17 of group II). Similar abnormalities affecting chromosome 7 were seen in three of 17 (17.7%) of low-grade NHL with t(14;18) and six of 71 (8.5%) NHL without t(14;18) in our series.

DISCUSSION

Recent studies have established a strong correlation between t(14;18) and follicular small cleaved cell lymphoma.^{6,9} However, this translocation has been noted in lymphomas lacking both a follicular pattern and a small cleaved cell component. A previous report described two cases of nodular poorly differentiated lymphocytic lymphoma that transformed to L3 acute leukemia and exhibited t(14;18).¹⁰ In addition, we have identified in the published literature nine cases of lymphomas carrying the t(14;18) that showed evidence of histological grade transformation.^{3,4,9} In this report we describe seven cases of lymphoma with evidence of transformation from low grade to intermediate or high grade and an additional 11 cases that presented with a diffuse growth pattern, a large cell component, or both, all of which displayed t(14;18). These data, although underscoring the association between t(14;18) and small cleaved cell follicular histology, also demonstrate that such lymphomas retain the t(14;18) when they transform to a higher grade.

We have identified recurrent chromosome abnormalities affecting chromosomes 6 and 7 in addition to t(14;18) in association with intermediate and high-grade histology in our series of 18 cases. Examination of the published karyotype data of 11 cases of low-grade lymphomas that have undergone transformation and 18 cases of intermediate to high-grade NHL all of which carried t(14;18), revealed some of the same changes that we have described here.^{2,5,9,10} Thus, loss of chromosome 6 or del(6q) was seen in two cases of the former group and five cases of the latter group (7/29; 29.1%), and more than two copies of chromosome 7 or duplication of 7q were seen in five cases of the former group and eight cases of the latter group (13/29; 44.8%). Thus, these data are in agreement with our results.

The highly nonrandom nature of chromosome 6 and 7 changes that we identified here strongly suggest a role in determining the ultimate grade of t(14;18)-carrying lymphomas. Interestingly, these same abnormalities have recently been shown by Yunis et al⁹ to correlate with an aggressive course when accompanying t(14;18) in follicular lymphomas.⁹ Thus, the additional abnormalities described here can serve as indicators of grade transformation and patient prognosis.

Evidence for gene deregulation in lymphomas mediated by chromosome rearrangement comes from data on *c-myc* expression in t(8;14)(q24;q32) associated with Burkitt's lymphoma.¹² The constitutive function of *c-myc* appears to be cell cycle regulation, and its deregulated expression under the influence of the immunoglobulin locus leads to escape of growth regulation by the cell.¹³ The gene, *bcl-2*, situated at 18q21 is frequently rearranged in t(14;18) and has been suggested to undergo deregulation.^{7,8,14,15} Its constitutive function is unknown; however, it is commonly rearranged in follicular lymphomas,^{14,15} and it has been suggested to undergo deregulation in t(14;18).^{7,8,14,15} Histologically, the follicular lymphomas express an architecture reminiscent of a normal lymph node. Therefore, it is attractive to speculate that *bcl-2* also may play a role in cell cycle regulation, but with lesser efficiency than *c-myc*, or alternatively, that it is a locus associated with differentiation of lymph node architecture. In either case, t(14;18)-carrying tumors with a documented history of grade transformation as well as those presenting as apparent de novo intermediate or high-grade lesions represent an overriding of the deregulated function of *bcl-2*. Whether the basis for this is a secondary activation of *c-myc*, as suggested by the phenotype, or deregulation or dosage changes of genes on the additional chromosomes that we now show to exhibit nonrandom abnormalities is unknown at present.

Chromosomes 6 and 7 are of interest from the point of view of the genes that they carry. The frequent site of deletion of 6q observed in these tumors corresponds to the site of the cellular oncogene *c-myc*.¹⁶ Loss of heterozygosity at key loci have been shown to be critical steps in the origin of tumor types such as retinoblastoma and Wilms' tumor.¹⁷ Chromosome 7 perturbations similar to the ones that we describe here have been noted in a variety of tumors originating from diverse cell types.¹⁸⁻²¹ The epidermal growth factor receptor gene has been localized to this chromosome.¹⁶ Growth factors have been shown to have synergistic effects on cellular oncogenes; for example, platelet-derived growth factor increases the level of *c-myc* expression in NIH3T3 cells.²² Dosage alteration of one or more growth-regulating genes on chromosome 7 may exert similar effects on *bcl-2* or *c-myc* that result in expression of higher-grade phenotypes by t(14;18)-carrying lymphomas.

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CML Patients in Blast Crisis Have Breakpoints Localized to a Specific Region of the BCR

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Chronic myelogenous leukemia (CML) is associated with the Philadelphia (Ph) chromosome, which results from a reciprocal translocation between chromosomes 9 and 22. This activates the *abl* oncogene by moving it from chromosome 9 and combining it with sequence located on chromosome 22. The new fusion gene, with chromosome 22 sequence at its 5' end and chromosome 9-*abl* sequence at its 3' end, generates a new messenger RNA (mRNA) and protein that are implicated in the pathogenesis of CML. The breakpoint near the *c-abl* locus on chromosome 9 can occur within a large area. In contrast, the breakpoints on chromosome 22 are concentrated within a 6 kilobase (kb)

region termed the breakpoint cluster region (bcr). This study was designed to determine whether chronic-phase and blast crisis patients had identifiable differences in the structure of their Ph chromosomes. Restriction mapping of the chromosome 22 translocation breakpoints performed for 26 patients showed that the breakpoints of eight of the nine patients in blast crisis were in the 3' portion of the bcr, whereas the breakpoints in the 17 patients in the chronic phase were clustered in the 5' portion of the bcr. This suggests a strong correlation between a 3' bcr breakpoint and blast crisis in CML.

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CHRONIC MYELOGENOUS LEUKEMIA (CML) occurs primarily in the adult population and has two distinct phases.¹⁻³ Initially a chronic phase is present that is characterized by an accumulation of mature granulocytes. This lasts for an average of 2 to 3 years and often responds well to single-agent chemotherapy. Subsequently, most patients enter what is termed blast crisis, which is similar to adult acute leukemia but even less responsive to therapy.⁴⁻⁶ Some patients pass through an additional accelerated phase with gradual worsening of the disease before entering blast crisis. CML was uniformly fatal before the recent successes with bone marrow transplantation.⁷⁻¹¹

The Philadelphia (Ph) chromosome was the first human chromosomal abnormality found associated with a malignancy and is still the most consistently detected one.^{12,13} Though initially described as a minute chromosome 22, the Ph chromosome was later shown to be the result of a reciprocal translocation between chromosomes 9 and 22.¹⁴ The human protooncogene *abl* (*c-abl*) is located on the terminal portion of chromosome 9.¹⁵ The *c-abl* gene is the human homologue of the transforming gene of the Abelson murine leukemia virus (*v-abl*).¹⁶ This gene is a member of the *src*-family of tyrosine kinase proto-oncogenes.

The translocation moves the *c-abl* oncogene from its normal location on chromosome 9 to the Ph chromosome.^{17,18}

The *abl* sequences are moved into the middle of a gene located on chromosome 22 that has been named *bcr*.^{19,20} This creates a new fusion gene that has *bcr* sequence at its 5' end and *abl* sequence at its 3' end.^{21,22} A unique new messenger RNA (mRNA) species 8.5 kilobases (kb) in length is transcribed from this gene, and a new protein of 210 kilodaltons (kd) is translated from the 8.5-kb mRNA.²³⁻²⁹ This new *bcr/abl* fusion protein has a tyrosine kinase activity that is greatly increased in comparison with the presently identified *abl* protein.³⁰

The breakpoints on chromosome 9 occur 5' to the *v-abl* homologous region. The distance from the *v-abl* homologous region to the chromosome 9 breakpoint ranges from 6 kb to greater than 100 kb.^{17,19,31} The part of *c-abl* moved to the Ph chromosome includes at least the *v-abl* homologous region and a small exon located just 5' to it. In the cases examined to date, this small *c-abl* exon forms the mRNA junction between *bcr* and *abl* sequence in the *bcr/abl* fusion gene transcript.^{21,22}

The fragment of chromosome 22 that forms the Ph chromosome is 5' to the *abl* sequences and joins these sequences from a breakpoint within the 6-kb breakpoint cluster region (bcr, providing the name *bcr* for the surrounding gene). From 20% to 30% of the patients delete chromosome 22 sequence located 3' to the bcr during the translocation.³² Previously reported breakpoints have been concentrated in the 5' portion of the bcr, without an indication of the clinical status of the patients.^{19,20} This study examined 26 CML patients, including nine patients in blast crisis, and found that there is a striking difference in the location of the chromosome 22 breakpoints depending upon the phase of the leukemia. Eight of nine patients studied in blast crisis have breakpoints in the 3' end of the bcr, whereas the breakpoints of the 17 patients studied in chronic phase are clustered in the 5' bcr.

MATERIALS AND METHODS

The 26 patients included in this study were all diagnosed as having Ph chromosome-positive CML when they were in the chronic phase of the leukemia. This study does not include patients who presented with acute lymphocytic leukemia and were subsequently found to have a Ph chromosome on karyotype. The initial blood sample was obtained from 9 of the patients when they had progressed to blast

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crisis (>30% blasts on bone marrow examination). The initial blood samples of the remaining 17 patients were obtained while in chronic phase. Three of the patients, MK, TH, and FP, who were in chronic phase at the time of their initial sample, progressed into blast crisis during the course of the study.

The isolation of DNA from total peripheral WBC used methods described in previous publications from this laboratory.³¹ Previously isolated cloned fragments of chromosome 22 were used to isolate a clone from a human cosmid library from which the probes used in this study were subcloned.³² The bcr was divided into five zones based upon the published restriction map (Fig 1).¹⁹ The probes were used to examine Southern blots of 26 CML patients and allowed the assignment of each patient's breakpoint to one of the zones within the bcr.

Restriction analysis was used to localize the breakpoints to one of the bcr zones. If a probe detects only the normal restriction band with a particular enzyme, then the translocation breakpoint is not within the fragment. By using different probes along with the indicated restriction enzymes it is possible to localize the region containing the breakpoint. The use of multiple restriction enzymes and probes minimizes the problem of restriction polymorphisms that lead to misinterpretations of the data.

RESULTS

The restriction data used to make the breakpoint location assignments are presented in Table 1, and the restriction mapping for two of the patients is shown in Figs 2 and 3. In the table of restriction data, patients with breakpoints in zones 1, 2, or 3 have been grouped together, as have patients with breakpoints in zones 4 and 5, which divides the patients into two groups depending upon whether their breakpoint is 5' or 3' of the central *Hind*III site. Patients were required to have at least 30% blasts in their bone marrow to be considered in blast crisis.⁵

Figure 2 illustrates the way in which the breakpoint for

patient GP was assigned to zone 3. Using probe 1 with a *Hind*III digest showed that the breakpoint was not located in zone 1. Probe 3 with a *Hind*III digest showed that the breakpoint was not located within zones 4 or 5. The breakpoint was detected when probe 3 was used with a *Bam*HI digest, which indicated that it was located within zone 3. The pattern of abnormal bands seen with probes 2 and 3 defines the breakpoint assignment. The fact that probes 1 and 3 identify different abnormal *Bgl*II fragments is most consistent with the interpretation that the Ph chromosome junction fragment is seen with probe 1 and the 9q+ chromosome junction fragment is seen with probe 3 (see the legend to Fig 2B for further details). A similar analysis using the same probes and restriction digests plus probes 2 and 4 allows the localization of the breakpoints for the other patients with 5' breakpoints.

The localization of the breakpoints in most of the patients is straightforward. In a few of the patients it is necessary to make additional assumptions to determine the best breakpoint assignment. In patient CM, abnormal fragments with *Bgl*II, *Hind*III, and *Bam*HI support assignment to zone 1. For patient Std, the breakpoint assignment to zone 3 is supported by data with the enzymes *Hind*III, *Bam*HI, and *Eco*RI. The *Bgl*II fragments from the Ph chromosome of patients CM and Std are presumably the same size as the normal *Bgl*II fragment. Similarly, two of the *Hind*III bands detected by probe 2 in patient MP are presumed to be of identical size, and the abnormal *Bam*HI fragment from the 9q+ chromosome of patient RL is the same size as the normal fragment. For patient Wi, the finding of two abnormal fragments with the enzyme *Bgl*II and probe 1 strongly supports a breakpoint assignment to zone 1, though the abnormal *Hind*III band with probe 2 would suggest a breakpoint in zone 2. To justify the breakpoint assignment to

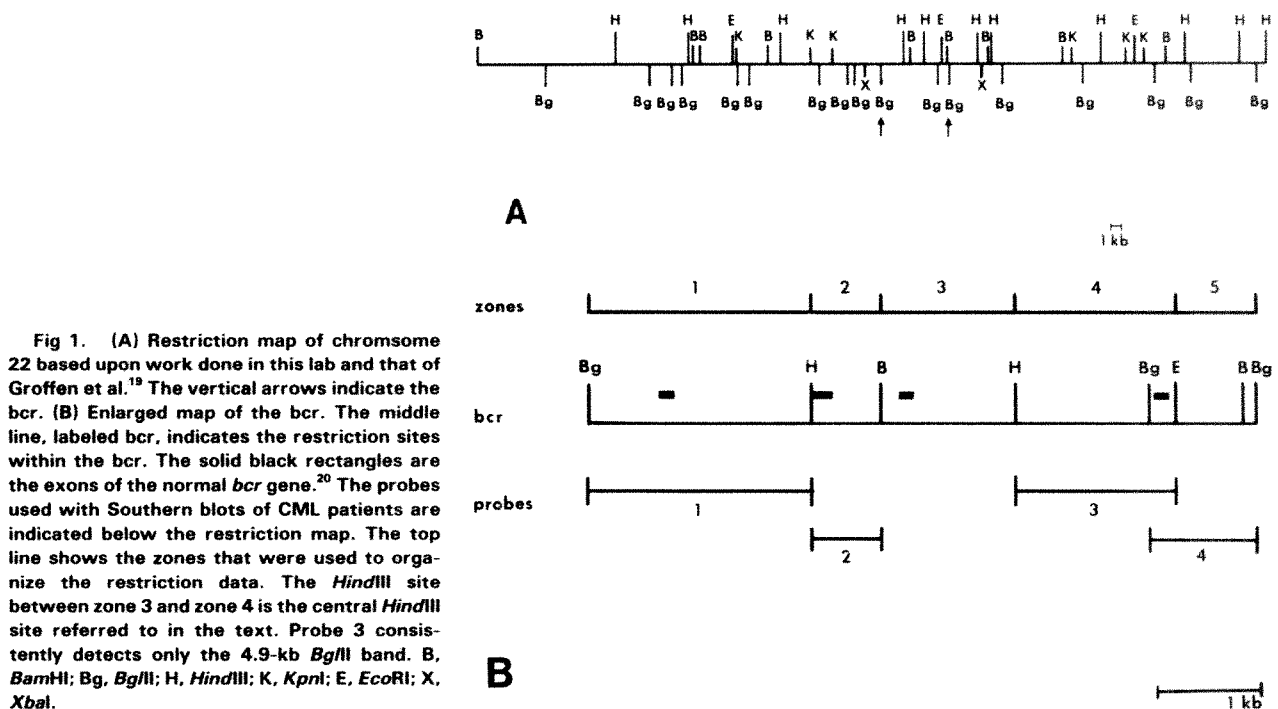


Table 1. Restriction Data

Patient	Duration of Chronic Phase to Date	Zone of Breakpoint
5' breakpoints		
GP (ch)	3 yr	3
Br (ch)	2 yr	2
RL (ch)	4 yr	3
MP (ch)	2.5 yr	2
Go'r (ch)	3 yr	3
HO (ch)	3 yr	3
KG (ch)	6 mo	2
HT (ch)	4 mo	2
NH (ch)	2 mo	3
ET (ch)	1 yr	2
JF (ch)	3 yr	3
CM (ch)	2.5 yr	1
Std (bl)	5 yr	3
Wi (ch)	4 yr	1
RC (ch)	4 yr	1
Length of Chronic Phase		
3' breakpoints		
MK (ch)†	3 mo	4
TH (ch)†	2.5 yr	4
FP (ch)†	3 yr	4
Ba (bl)	3.5 yr	4
Bo (bl)	3 yr	4
Al (bl)	2 yr	4
Stn (bl)	1.5 yr	4
Me (bl)	3 yr	4
RT (bl)	2.5 yr	4
Mi (bl)	2 yr	4
Ku (bl)	4 yr	4
Probe 1		
	BglII	HindIII
5' breakpoints		
GP (ch)	4.9/6.5*	10
Br (ch)		10
RL (ch)	4.9/4.0*	10
MP (ch)	4.9/6.8*	10
Go'r (ch)	4.9/8.2*	10
HO (ch)	4.9/7.3*	10
KG (ch)	4.9/2.4*	10
HT (ch)	4.9/16*	10
NH (ch)	4.9	10
JF (ch)	4.9/8.1*	10
ET (ch)	4.9/3.2*	10
CM (ch)	4.9/5.8*	10/22*
Std (bl)	4.9	10
Wi (ch)	4.9/7.9*/2.4*	
RC (ch)	4.9/7.4*	10/16*
3' breakpoints		
MK (ch)	4.9/9.4*	10
TH (ch)	4.9/7.8*	10
FP (ch)	4.9/3.6*	10
Ba (bl)	4.9/10*	
Bo (bl)	4.9/6.6*	10
Al (bl)	4.9/4.0*	10
Stn (bl)	4.9/6.8*	10
Me (bl)	4.9/6.8*	10
RT (bl)	4.9/11*	10
Mi (bl)	4.9/7.4*	10
Ku (bl)	4.9	10
Probe 2		
	BglII	HindIII
5' breakpoints		
GP (ch)	4.9/6.5*	1.8/4.0*
Br (ch)	4.9/12*	1.8/1.3*
RL (ch)	4.9/4.0*	1.8/3.6*
MP (ch)	4.9/4.0*/6.8*	1.8/13.5*
Go'r (ch)	4.9/8.2*	1.8/1.4*
HO (ch)	4.9/7.3*	1.8/1.3*
KG (ch)	4.9/3*/2.4*	1.8/3.9*/8*

Table 1. Restriction Data (Cont'd)

Patient	Duration of Chronic Phase to Date	Zone of Breakpoint
Probe 2		
	BglII	HindIII
5' breakpoints		
HT (ch)	4.9/16*/4.4*	1.8/2.6*
NH (ch)	4.9	1.8/2.6*
JF (ch)	4.9/4.5*	1.8/8.7*/3.9*
ET (ch)	4.9/3.2*	1.8/7.7*
CM (ch)	4.9	
Std (bl)	4.9	1.8/23.6*
Wi (ch)	4.9/7.9*	1.8/2.9*
RC (ch)	4.9	
3' breakpoints		
MK (ch)	4.9/9.4*	1.8
TH (ch)	4.9/7.8*	1.8
FP (ch)	4.9/3.6*	1.8
Ba (bl)	4.9/10*	1.8
Bo (bl)	4.9/6.6*	1.8
Al (bl)	4.9/4.0*	1.8
Stn (bl)	4.9/6.8*	1.8
Me (bl)	4.9/6.8*	1.8
RT (bl)	4.9/11*	1.8
Mi (bl)	4.9/7.4*	1.8
Ku (bl)	4.9	1.8
Probe 3		
	BglII	BamHI
5' breakpoints		
GP (ch)	4.9/7.3*	3.2/2.5*
Br (ch)	4.9/8.4*	3.2
RL (ch)	4.9/6.9*	3.2
MP (ch)	4.9/4.0*	3.2
Go'r (ch)	4.9/7.2*	3.2/5.3*
HO (ch)	4.9	3.2
KG (ch)	4.9/3*	3.2
HT (ch)	4.9/4.4*	3.2
NH (ch)	4.9/4.7*	
JF (ch)	4.9/4.5*	3.2
ET (ch)	4.9	3.2
CM (ch)	4.9	3.2
Std (bl)	4.9	3.2/2.9*
Wi (ch)	4.9/7.9*	3.2
RC (ch)	4.9	3.2/6.2*
3' breakpoints		
MK (ch)	4.9/9.4*/2.4*	3.2/6.3*/22*
TH (ch)	4.9/7.8*	3.2/4.2*
FP (ch)	4.9/11*	
Ba (bl)	4.9/10.0*/2.1*	3.2/14.7*
Bo (bl)	4.9/6.7*	3.2/11.8*/5.4*
Al (bl)	4.9/4.0*/1.6*	
Stn (bl)	4.9/6.1*	
Me (bl)	4.9/6.8*/1.4*	
RT (bl)	4.9/11*	
Mi (bl)	4.9/7.5*	3.2/8*/26*
Ku (bl)	4.9/7.1*	3.2/16*
Probe 4		
	BamHI	BglII
5' breakpoints		
GP (ch)		3.2/2.5
Br (ch)		3.2/2.5
RL (ch)		3.2/2.5
MP (ch)		3.2/2.5
Go'r (ch)		3.2/2.5/5.3*
HO (ch)		3.2/2.5
KG (ch)		3.2/2.5
HT (ch)		3.2/2.5
NH (ch)		3.2/2.5/17.7*
JF (ch)		3.2/2.5
ET (ch)		3.2/2.5
CM (ch)		3.2/2.5
Std (bl)		3.2/2.5/2.9*
Wi (ch)		3.2/2.5
RC (ch)		3.2/2.5

Table 1. Restriction Data (Cont'd)

Patient	Duration of Chronic Phase to Date		Zone of Breakpoint	
	Probe 4			
	<i>Bam</i> HI	<i>Bgl</i> II	<i>Hind</i> III	<i>Eco</i> RI
3' breakpoints				
MK (ch)	3.2/2.5/22*		4.2/ 5.9*	16.7
TH (ch)	3.2/2.5		4.2	
FP (ch)	3.2/2.5/11.4*			
Ba (bl)	3.2/2.5/14.7*			
Bo (ch)	3.2/2.5/5.4*/11.8*	1.0	4.2/ 3.6*	
Al (ch)			4.2/ 4.6*	
Stn (ch)	3.2/2.5/3.4*		4.2/ 5.8*	
Me (ch)	3.2/2.5/21.4*	1.0	4.2/ 19.2*	
RT (ch)	3.2/2.5	1.0	4.2/ 11.9*	
Mi (ch)	3.2/2.5/8*		4.2/ 7.4*	
Ku (ch)	3.2/2.5/1*	1/7.1*	4.2/ 9.1*	

Patients are grouped by breakpoint location either 5' (zones 1, 2, and 3) or 3' (zones 4 and 5) to the central *Hind*III site in Fig 1. The length of time that a patient had been in chronic phase at the time of sample is indicated for patients in chronic phase. The duration of the chronic phase is indicated for patients in blast crisis at the time of their first sample. Table 1 also indicates the zone of the breakpoint deduced from the restriction data and summarized in Table 2. Numbers indicate the size in kilobases of the bands detected on Southern blots with the indicated restriction enzyme and probe.

Abbreviations: ch, in chronic phase at the time of first sample; bl, in blast crisis at the time of first sample.

*Abnormal band

†Patients who were in chronic phase and entered blast crisis during the time of observation.

zone 1 for the patient RC, it is necessary to assume that there is a small deletion and the abnormal *Bam*HI and *Eco*RI fragments with probe 3 are from the 9q+ chromosome.

The informative restriction mapping for a patient in blast crisis with a 3' *bcr* breakpoint is illustrated in Fig 3. The general analysis is similar to that for patient GP, though for patient MK the diagnostic finding of two abnormal fragments with probe 3 indicates that the breakpoint lies in zone 4 (see the legend to Fig 3B for details). The assignments for the other patients with 3' breakpoints, except for Stn and Ku, are also based on the finding of two abnormal fragments with either probe 3 or probe 4. For Stn, *Bgl*II with probes 1 and 2 shows an abnormal fragment from the Ph chromosome, thereby indicating the presence of a translocation, whereas *Hind*III with probes 1 and 2 is normal, which indicates that the breakpoint is not within zones 1, 2, or 3. Probe 4 detects an abnormal fragment with *Bam*HI and *Hind*III, and probe 3 detects an abnormal *Bgl*II fragment from the 9q+ chromosome, which localizes the breakpoint to zone 4. Patient Ku produces normal fragments with probes 1 and 2 and then abnormal fragments with probes 3 and 4 with *Bgl*II, *Bam*HI, *Eco*RI, and *Hind*III. The abnormal *Eco*RI band with probe 3 supports a breakpoint assignment to zone 4, whereas the other abnormal bands are consistent with a breakpoint in zone 4 or zone 5.

In Table 2 the patients are categorized by the location of their breakpoint and their clinical condition at the time of their first blood sample. The patients in chronic phase had breakpoints distributed throughout the *bcr* but concentrated

in the 5' portion, zones 2 and 3. With one exception, all the patients in blast crisis had breakpoints located 3' to the central *Hind*III site, zone 4. In addition, each of the three patients in chronic phase with 3' breakpoints (MK, TH, FP, Tables 1 and 2) have progressed into blast crisis, one within 3 months of initial presentation of disease and the others after being in chronic phase 2½ and 3 years, respectively. None of the chronic-phase patients with 5' breakpoints have entered blast crisis. These results strongly support the hypothesis that a 3' breakpoint is associated with the development of blast crisis.

DISCUSSION

This study demonstrates a strong correlation between a 3' *bcr* breakpoint and either being in blast crisis or evolving toward it. These data raise two issues: (a) By what mechanism could a more 3' breakpoint have an effect upon individual patients, and (b) What is the relationship between the groups of patients identified as having a 3' or a 5' breakpoint?

One mechanism by which genes with 3' breakpoints could have effects different from genes with 5' breakpoints would be by producing increased amounts of a structurally

Table 2. Summary of Breakpoint Data for 26 CML Patients

	Zones				
	1	2	3	4	5
Chronic	RC† CM Wi	Br MP ET† KG HT	NH Go'r HO† GP JF RL	MK* TH† FP	
	Pts/kb	(1.7)	(7.9)	(5)	(2.5)
Blast			Std	Ba Bo Al Stn Me RT Mi Ku	
	Pts/kb		(0.83)	(6.7)	

The zones are as defined in Fig 1. The initials below each zone heading identify the patients with breakpoints within that zone. As indicated at the left, the patients are grouped into either chronic phase or blast crisis, depending upon their clinical status at the time of their initial sample. Seventeen patients were in chronic phase, and nine were in blast crisis at the time of their initial sample. The numbers below the initials indicate the number of patients with breakpoints within each zone, corrected for the fact that the zones are of different sizes. The asterisk next to MK indicates that this patient entered blast crisis within 3 months of presentation. The other patients who were in chronic phase but with 3' breakpoints at the time of their presentation entered blast crisis after 2½ years (TH) and 3 years (FP). No other patients in this group have entered blast crisis during the period of observation (from 9 months to 4 years). The daggers indicate patients with deletions of chromosome 22 sequence. The group of patients in blast crisis were approximately two-thirds myeloid and one-third lymphoblastic.

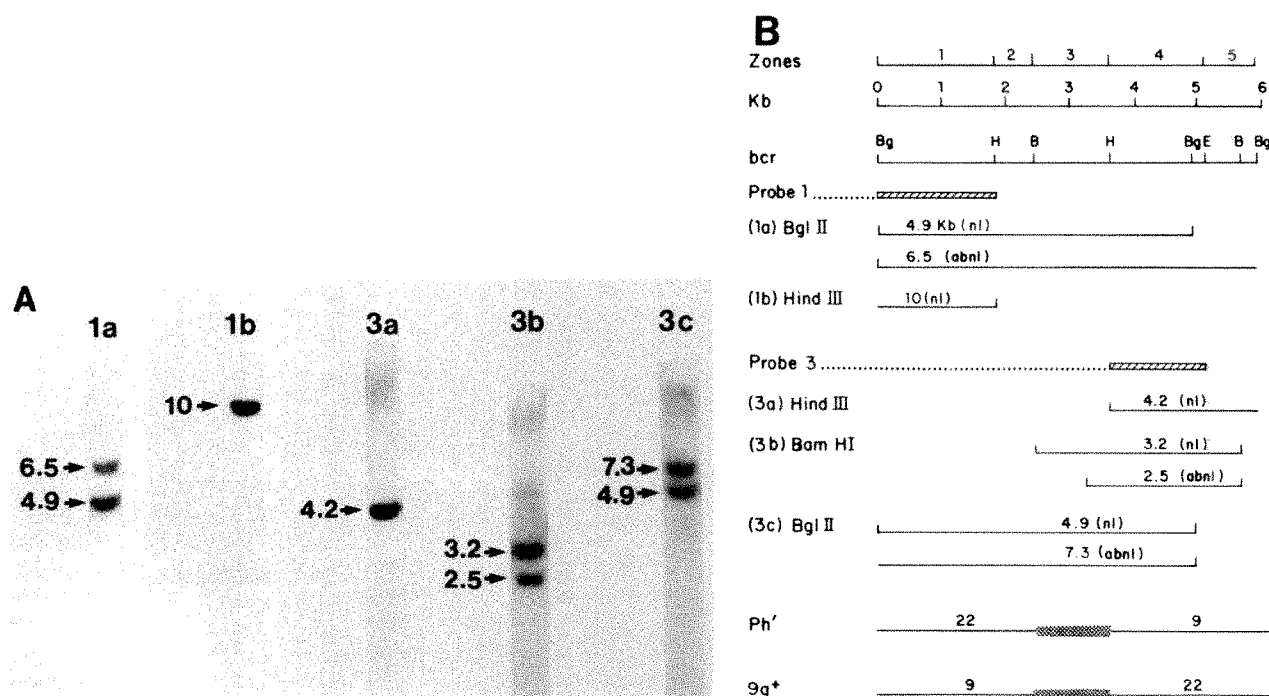


Fig 2. (A) Selected Southern blots used to localize the breakpoints in patient GP. These blots as well as other blots of this patient are listed in Table 1. The blots are from different gels, so band position does not correlate with size in kilobases from one gel to another. The interpretation of the blots is shown schematically in Fig 2B. Lane 1a, the probe was probe 1, and restriction enzyme was *Bgl*II. The normal 4.9-kb band is seen as well as an abnormal 6.5-kb band. This abnormal 6.5-kb *Bgl*II band is from the Ph chromosome (see the following and the legend to Fig 2B). Lane 1b, probe 1, *Hind*III, normal 10-kb band; lane 3a, probe 3, *Hind*III, normal 4.2-kb band; lane 3b, probe 3, *Bam*HI, normal 3.2-kb band, abnormal 2.5-kb band; lane 3c, probe 3, *Bgl*II, normal 4.9-kb band, abnormal 7.3-kb band. The abnormal 7.3-kb *Bgl*II band is from the 9q+ chromosome. (B) Restriction mapping of the GP breakpoint. The restriction fragments shown correspond to those seen in the Southern blots in Fig 2A (lane 1a represents Southern blot 1a, etc). The restriction enzyme and probe used for each blot are indicated on the left. Probe 1 with *Bgl*II (1a) shows a normal band of 4.9 kb and an abnormal band of 6.5 kb, which indicates that there is a translocation breakpoint within the 4.9-kb *Bgl*II fragment. Probe 1 with a *Hind*III digest (1b) generates a single normal band of 10 kb, which indicates that the breakpoint is 3' to this fragment in zones 2, 3, or 4. Probe 3 with *Hind*III (3a) shows only the normal 4.2-kb fragment, thereby indicating that the breakpoint is not within this fragment, which includes zones 4 and 5. A *Bam*HI digest probed with probe 3 (3b) shows the normal 3.2-kb band and an abnormal 2.5-kb fragment, thereby indicating that the translocation breakpoint lies within the *Bam*HI fragment. Since zones 4 and 5 are normal, the breakpoint must be in zone 3. Probe 3 with *Bgl*II (3c) again generates a 4.9-kb normal fragment and a 7.3-kb abnormal fragment. The abnormal *Bgl*II fragment seen with probe 3 is from the 9q+ chromosome (illustrated at the bottom of the figure). Bg, *Bgl*II; H, *Hind*III; B, *Bam*HI; E, *Eco*RI.

unchanged *bcr/abl* protein, which could result from either an increase in the transcription of the fusion gene or from a prolongation of the $t_{1/2}$ of the *bcr/abl* mRNA.³³ The location of the breakpoint could also disrupt transcriptional regulation and produce an inappropriate amount of fusion mRNA and protein at critical points during the life cycle of the leukemic cells rather than an increase in the absolute amount of fusion gene mRNA.

The *bcr/abl* fusion protein in patients with a 3' *bcr* breakpoint might also be qualitatively different from the protein present in patients with a more 5' breakpoint. This could result from the inclusion of an additional exon in the mRNA transcript of the patients with 3' breakpoints. For example, the presence of exon 3 in the Ph chromosome-derived fusion transcript may be important in explaining the clustering of blast crisis breakpoints. Exon 4 of the *bcr* lies at the extreme end of the *bcr* zone that contains most of the blast crisis breakpoints, so it is unlikely that the difference is caused by the simple presence or absence of exon 4.²⁰ An altered mRNA and protein could also result from the use of a cryptic splice site in the large intron in patients with a 3'

breakpoint. This novel protein would then either have an altered kinase activity, a different substrate specificity, or a combination of both characteristics.

Differential splicing of exons 2 and 3 within the *bcr* would also generate differences between the total *bcr/abl* fusion protein activity in individual patients. A recent report examining two CML cell lines and a small number of patients with 3' breakpoints detected splicing from both exon 2 as well as exon 3 to the common *c-abl* exon, thereby generating two species of mRNA in a single patient: [*bcr*]-exon 2-exon 3-*abl*, and also [*bcr*]-exon 2-*abl*.³⁴ Fusion proteins containing both *bcr* exon 2 and exon 3, in comparison to fusion proteins containing only exon 2, could well have a different kinase activity or substrate specificity.³⁵ The distance between a 3' breakpoint and the exon 3 splice donor site may affect the proportion of exon 2-*abl* splicing compared with exon 3-*abl* splicing. There may also be a difference in the $t_{1/2}$ of the protein or mRNA, depending upon whether exon 3 is included in the mRNA transcript. There are clearly a number of hypothetical mechanisms by which a different breakpoint could produce a phenotypic difference. Which of

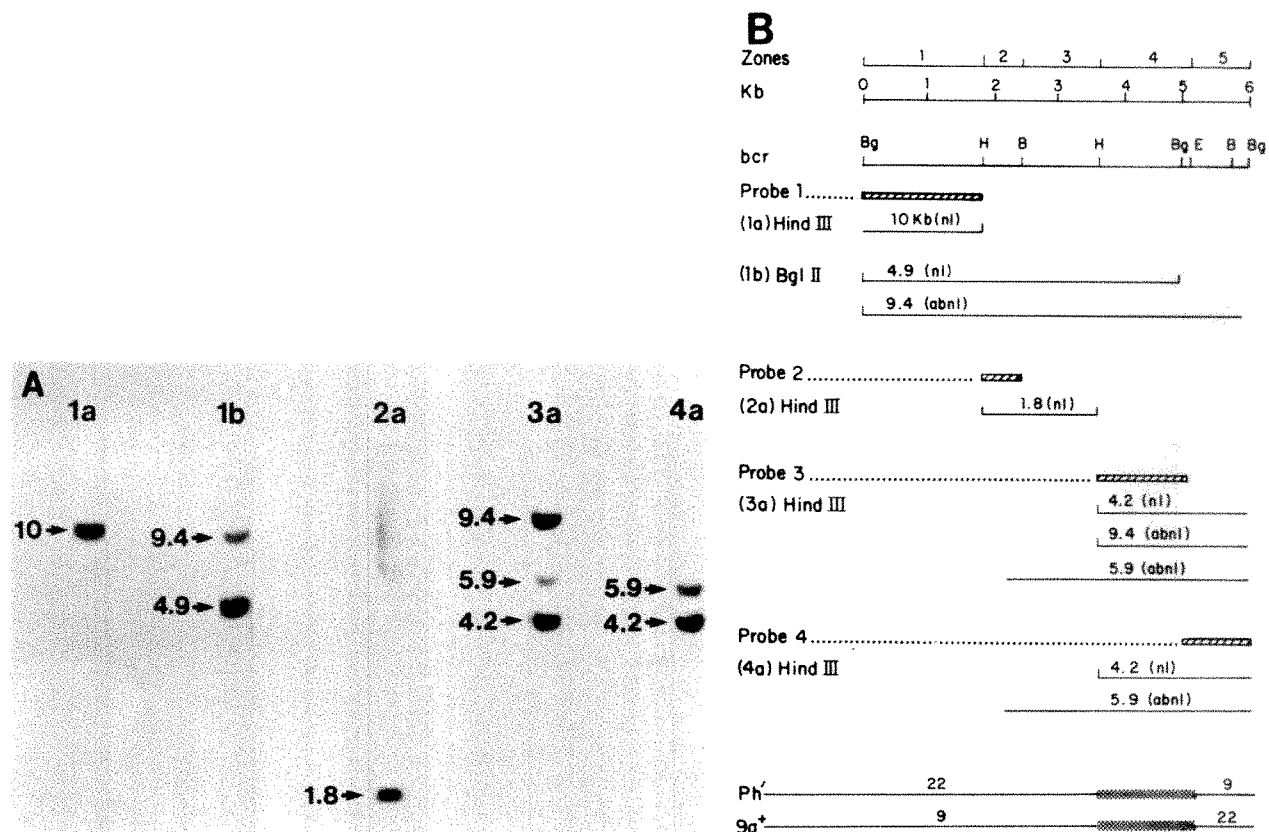


Fig 3. (A) Selected Southern blots used to localize the breakpoint in patient MK with a 3' breakpoint. Lane 1a, probe 1, restriction enzyme *Hind*III, normal band of 10 kb; lane 1b, probe 1, *Bgl*II, normal 4.9-kb band, abnormal 9.4-kb band; lane 2a, probe 2, *Hind*III, normal 1.8-kb band; lane 3a, probe 3, *Hind*III, normal 4.2-kb band, abnormal 5.9-kb and 9.4-kb bands; lane 4a, probe 4, *Hind*III, normal 4.2-kb band, abnormal 5.9-kb band. (B) Restriction mapping of the MK breakpoint. The restriction fragments in the illustration correspond to the Southern blots in 3A. The restriction enzymes and probes used are indicated on the left. Probe 1 with the restriction enzyme *Hind*III (1a) shows only the normal 10-kb fragment, which indicates that the breakpoint is not within this fragment, including zone 1. Probe 1 with *Bgl*II (1b) detects a normal 4.9-kb fragment and an abnormal 9.4-kb fragment. The abnormal 9.4-kb fragment is presumably from the Ph chromosome, with chromosome 22, sequence 5', and chromosome 9, sequence 3'. The translocation breakpoint must lie within the 4.9-kb *Bgl*II fragment in zone 2, 3, or 4. Probe 2 with *Hind*III (2a) detects a single normal 1.8-kb fragment, which indicates that the breakpoint does not lie within zone 2 or 3. Probe 3 with *Hind*III (3a) detects a normal 4.2-kb fragment and two abnormal fragments, of 5.9 and 9.4 kb. The fact that probe 3 detects two abnormal fragments, one from the Ph chromosome and one from the 9q+ chromosome, indicates that the translocation breakpoint lies within the *Hind*III-*Eco*RI fragment, which is the probe itself, zone 4. Probe 4 with *Hind*III (4a) detects a normal 4.2-kb fragment and the abnormal 5.9-kb fragment seen with probe 3. Since probe 4 detects only one abnormal *Hind*III fragment, this probe must be located 3' of the translocation breakpoint. The chromosome 22 sequence located 3' of the translocation breakpoint is part of the 9q+ chromosome, so the 5.9-kb abnormal fragment detected by both probe 3 and probe 4 contains the translocation junction fragment from the 9q+ chromosome. The deduced Ph and 9q+ chromosomes are shown at the bottom, with the broken line indicating the breakpoint region.

these mechanisms actually has an effect upon patients with CML will be learned only from more extensive in vitro molecular studies and additional careful analysis of individual patients.

The next question to consider is how the patients with 5' breakpoints are related to the group with 3' breakpoints. Since the majority of patients with CML terminate their disease in blast crisis, it is reasonable to assume that at least a large portion of the chronic-phase patients with zone 2 and 3 breakpoints will enter blast crisis. Some of these chronic-phase patients with 5' breakpoints may undergo a second 9,22 translocation resulting in another Ph chromosome with a 3' breakpoint in conjunction with the transformation of their leukemia. The most common abnormality for CML patients to develop as their disease progresses is a second Ph

chromosome, and in some cases this second Ph chromosome may represent an additional translocation with a 3' breakpoint (though cytogenetic data have suggested that the additional Ph chromosome often represents a duplication of the first).³⁶ We have not yet analyzed DNA from a patient with two Ph chromosomes, and the study reported here did not include patients with 5' breakpoints who were followed as their disease progressed from chronic phase into blast crisis. The possible clonal evolution of patients in chronic phase with 5' breakpoints can only be recognized by prospective studies that examine their breakpoints as the disease progresses. The only patients who progressed from chronic phase to blast crisis during the time this study was being performed were the three patients in chronic phase with 3' breakpoints.

Tumorigenesis is probably a multistep process requiring a number of alterations in the normal physiology of a cell before the malignant phenotype emerges. Having a more 3' bcr breakpoint appears to identify a group of CML patients in or soon to enter blast crisis. If entering blast crisis requires passing a certain threshold of *bcr/abl* fusion protein activity, then other mechanisms such as gene amplification or promoter mutation could also provide increased levels of *bcr/abl* activity.

The presence of a 3' bcr breakpoint appears to identify a group of patients who are at a higher biologic risk for entering blast crisis. A prospective study that determines the bcr breakpoint location for a group of chronic-phase patients and then follows these patients through the course of their disease is needed to properly evaluate the prognostic utility of the breakpoint location. In addition to prospectively studying

chronic-phase patients, it will be necessary to do an even more detailed analysis of the structure of the Ph chromosome translocation, the *bcr/abl* fusion gene transcripts, and the fusion protein itself. This knowledge may influence therapeutic decisions such as the use of earlier marrow transplantation in certain patients. In addition, if the 5' and 3' breakpoints differ in their activation of *abl*, this may provide new insights into the mechanism of action of both the normal *abl* gene and the *bcr/abl* fusion gene.

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minutes at room temperature and twice in $0.1 \times \text{SSC}$ and 0.1% SDS for 15 minutes at 68°C . After drying the blots were exposed to x-ray films with an intensifying screen at -70°C .

To check that equal amounts of RNA had been blotted, nitrocellulose sheets were stained with methylene blue. The integrity of blotted RNA was shown by hybridization with an actin gene probe (Dianova, Hamburg, FRG) nick-translated in the same manner as described for c-fos.

Immunophenotyping of separated cell populations. For immunocytochemical analysis of U-937 cells we applied the monoclonal antibodies Ki-M1 and Ki-M6,¹⁸ which were raised against separated lysosomes of stimulated U-937 cells and show a restricted reactivity with blood monocytes and macrophages. The immunoperoxidase reaction was performed according to Stein et al¹⁹ by applying a three-step immunoperoxidase method.

Immunoblot analysis. Nuclei of granulocytes were separated by mechanical disruption of cell membranes followed by centrifugation. The nuclei were resuspended in 0.5 mol/L NaCl solution to extract the fos protein as described elsewhere.²⁰ Fos protein was detected after electrophoretic separation of the nuclear extract and immunoblot using an antiserum raised against a β -gal-c-fos fusion protein as described.^{20,21}

RESULTS

c-fos expression in normal human blood cells. We used the c-fos gene as well as the v-fos gene for the detection of fos-transcripts in isolated cellular RNA; both probes yielded identical results. As shown in Fig 1, a 2.2-kb transcript of c-fos appeared in all granulocyte samples analyzed. In some lanes an additional band of 2.0-kb could be observed. This band could be due to an artificial hybridization to 18S rRNA or may be related to 2.0-kb r-fos gene transcripts as assumed by other authors.^{10,22} None of the analyzed BM or AM probes revealed a detectable transcriptional activity of c-fos. Expression of c-fos was also missing in the lymphocyte cell populations analyzed.

c-fos expression in U-937 cell line. As shown in Fig 2 U-937 cells could be induced to express c-fos after one hour of TPA exposure. Simultaneously, U-937 cells attached to the bottom of the culture vessel. This early induction of c-fos

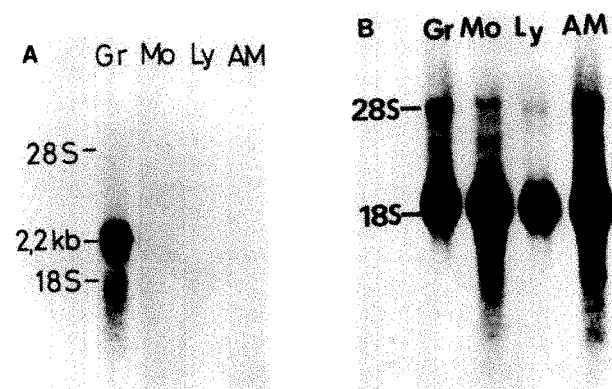


Fig 1. (A) Northern blots of total cellular RNA of granulocytes (G), blood monocytes (Mo), lymphocytes (Ly), and AM after hybridization with a labeled c-fos probe. Transcripts of 2.2-kb pairs length are only detectable in granulocytes. (B) Same probes as in (A) after hybridization with nick-translated actin-DNA. A corresponding transcript occurs in all probes, which indicates that RNA has not undergone degradation.

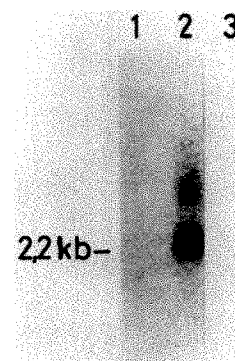


Fig 2. Northern blots of total cellular RNA of unstimulated U-937 cells (1), briefly TPA induced U-937 cells (2), and U-937 cells cultured for 72 hours following six hours' TPA stimulation (3) after hybridization with a labeled c-fos probe. Although unstimulated U-937 cells do not show detectable c-fos expression, transcripts appear in briefly (one hour) induced U-937 cells. After 72 hours when U-937 cells have acquired phenotypic properties of mature monocytes, c-fos transcription cannot be observed.

expression and adherence was not accompanied by induction of phenotypic properties typical for monocytes/macrophages as revealed by immunostaining with the monoclonal antibodies Ki-M1 and Ki-M6 specific for the monocyte/macrophage system (Table 1). Only U-937 cells induced for six hours with TPA and further cultured for 72 hours in normal medium lacking TPA transformed into macrophages and showed a strong activity for nonspecific acid esterase and positive reactivities with the monoclonal antibodies Ki-M1 and Ki-M6 (Table 1). RNA of U-937 cells committed in this way, however, did not contain detectable amounts of c-fos transcripts (Fig 2).

To compare the level of c-fos transcription we hybridized serial dilutions of granulocytic RNA and of RNA of briefly TPA induced U-937 cells to labeled c-fos probes. As shown in Fig 3 the level of c-fos transcription was about ten times higher in granulocytes than in briefly TPA induced U-937 cells.

Expression of fos protein in granulocytes. By using an antiserum raised against a β -gal-mouse-c-fos fusion protein the fos protein of approximately 60 kilodaltons (kd) is clearly demonstrable in protein extracts from nuclei of granulocytes as shown in Fig 4.

DISCUSSION

The fos oncogene is the transforming gene of the Finkel-Biskis-Jenkins osteosarcoma virus that was isolated from the

Table 1. Antigen Expression of Unstimulated and TPA-Stimulated U-937 Cells in Comparison With BM

	BM	U-937	U-937 2-h TPA	U-937 6-h TPA
Nonspecific acid esterase	+	(+)	(+)	+
OKM	+	+	+	+
KiM-1	+	—	—	+
KiM-6	+	—	—	+

After six hours of TPA stimulation U-937 cells acquired the immunophenotype of BM.

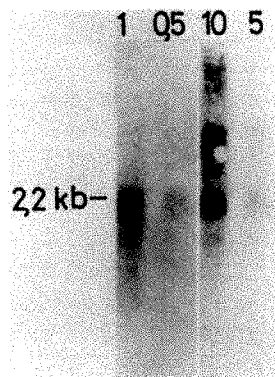


Fig 3. Northern blots of different amounts of total cellular RNA of granulocytes (1 μ g and 0.5 μ g) and briefly (one hour) TPA induced U-937 cells (10 μ g and 5 μ g) after hybridization with a labeled *c-fos* probe. *C-fos* expression in granulocytes is about ten times higher than in TPA-induced U-937 cells.

corresponding murine tumor.²³ The viral and cellular *fos* proteins differing at their COOH-termini are both located in the nucleus.^{17,24} Under normal conditions high levels of *c-fos* transcription have been found in human term fetal membranes²⁵ and in hematopoietic cell types present in the bone marrow of mice.²⁶ Several investigations have shown that the *c-fos* protooncogene is transcriptionally active in hematopoietic cell lines differentiating into macrophages.⁶⁻¹¹ The promyelocytic cell line HL-60, for example, characterized by a bimodal differentiation potency, revealed *c-fos* transcription merely after induction of monocytic but not granulocytic maturation. These data suggested a selective activation of the *c-fos* protooncogene within the monocytic pathway of the hematopoietic system.

In marked contrast to these results obtained by in vitro analysis with growth factor-independent cell lines, we found a different pattern of *c-fos* expression in purified normal human blood cells and macrophages. BM, AM, and lympho-

cytes did not exhibit detectable amounts of *c-fos* RNA, whereas high levels of *c-fos* transcription could be found in granulocytes (Fig 1). *Fos* protein was demonstrable in nuclei of granulocytes by immunoblot (Fig 4). U-937 cells expressed *c-fos* transcription transiently only after one hour of TPA stimulation (Fig 2), whereas U-937 cells stimulated by TPA over six hours and being phenotypically identical with monocytes/macrophages did not reveal *c-fos* transcription anymore.

The differences between the in vitro and in vivo observations may be due to the following mechanisms. First, it could be possible that chemical compounds inducing differentiation are also responsible for the activation of *c-fos* independently of the induction of monocytic differentiation. Thus, TPA leads to an activation of *c-fos* not only in HL-60 and U-937 cells but also in fibroblasts.⁸ Furthermore, *c-fos* transcripts could not be detected in HL-60 cells committed to monocytic maturation by the vitamin D₃ dihydroxy derivative 1.25-(OH)₂D₃.⁸ In this context, however, it remains obscure why the granulocytic differentiation of HL-60 cells induced by retinoic acid or dimethyl sulfoxide is not accompanied by a marked *c-fos* expression. An alternative explanation could be that BM precursors express *c-fos* transiently before leaving the bone marrow. This assumption would be in good agreement with the results presented in this study. Transcripts of the *c-fos* protooncogene appeared in U-937 cells one hour after TPA induction but were lost after acquisition of mature monocytic properties as shown by a positive immune reaction for the monoclonal antibodies Ki-M1 and Ki-M6 that recognize mature BM and their macrophage derivatives but not unstimulated U-937 cells (Table 1). A marked decrease of *c-fos* transcription in TPA-induced U-937 and HL-60 cells was also described by Mitchell et al¹⁰ and Sariban et al,⁹ which suggests that the gene product of *c-fos* is involved in the initiation of monocytic differentiation but not in its further maintenance. Recently, Mitchell et al²⁷ showed by analyzing TPA-resistant mutants of HL-60 that *c-fos* expression seems to be rather a circumstantial than a crucial event during myelomonocytic differentiation.²⁷

A rapid but transient expression of *fos* RNA was also observed in PC 12 cells treated with nerve growth factor²⁸ as well as in serum and PDGF-stimulated quiescent mouse fibroblasts.^{29,30} Obviously, the *c-fos* gene product provides a transducing signal that is evoked by different growth or differentiation factors in a cell-dependent manner and thus initiates selective activation of distinct cellular genes.³¹ The observation that *c-fos* is expressed constitutively in normal human granulocytes raises the question of its function in terminally differentiated cells. High levels of *c-fos* expression have been found during the late gestation phase in cells constituting the fetal membranes of the mouse and in the human placenta.^{25,32} Like the myelomonocytic precursor cells of the bone marrow, the cells of the trophoblast and decidua exhibit a highly proliferative capacity. It remains to be examined whether in all these cases the *fos* gene product interferes with the genetic mechanisms initiating terminal differentiation within these two rapidly proliferating cell populations. If so, it would be of great interest and clinical

Granulocytes Pellet Supern.

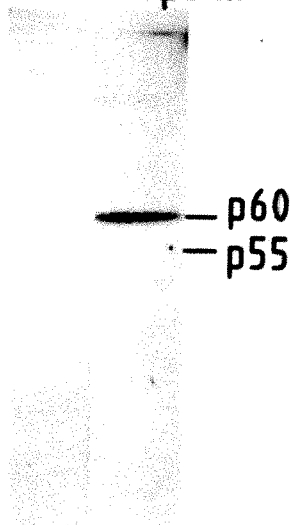


Fig 4. Immunoblot of proteins extracted from nuclei of granulocytes with 0.5 mol/L NaCl. A *fos* protein of approximately 60 kd is recognized in the supernatant of extracted nuclei, whereas the nuclei pellet after extraction does not show any immunoprecipitated protein. The 55-kd variant of the *fos* protein is neither detectable in the supernatant nor in the pellet.

relevance to ascertain the inducing signals leading to *c-fos* expression and hence terminal differentiation of myeloid cells. Furthermore, it must be concluded that the use of permanent cell lines as model systems for differentiation processes of hematopoietic cells can be misleading.

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Age-Related Deficiency of the Synthesis of Platelet Activating Factor by Leukocytes From Zellweger Patients

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Ca²⁺-ionophore A23187-induced synthesis of the alkoxyether lipid platelet activating factor (PAF) by leukocytes from Zellweger patients was undetectable in two patients studied at 3 and 4 weeks of age, reduced in a third patient studied at 2 months of age, and in the low normal range in four patients studied between 4 months and 5 years of age. We have previously reported that plasmalogen-type phosphatidylethanolamine (PE) levels of erythrocytes are reduced in Zellweger patients up to 20 weeks of age, but

normal in older patients. These levels were reduced in the three patients with abnormal PAF synthesis, and normal in the other four patients. The results suggest a close relationship between the age of the patients at sampling, and both the A23187-induced capacity of leukocytes to synthesize PAF and the plasmalogen PE levels in their erythrocytes.

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THE CEREBRO-HEPATO-RENAL syndrome of Zellweger is a rare disease with an autosomal recessive mode of inheritance. It is characterized clinically by a typical craniofacial dysmorphism, hepatomegaly, severe hypotonia and psychomotoric retardation, failure to thrive, and usually an early death as 80% of the patients die in the first 6 months of life.¹ In this syndrome disturbances of bile acid biosynthesis, very-long-chain fatty acid oxidation, and alkoxyether lipid biosynthesis have been reported,^{2,4} which may all be caused⁵ by the absence of ultrastructurally detectable peroxisomes in tissues of these patients.⁶ For instance, the activities of dihydroxyacetone phosphate acyltransferase (DHAP-AT) and alkyl-DHAP synthase, two enzymes involved in the biosynthesis of alkoxyether lipids, and shown to be localized in (micro)peroxisomes of rodent brain and liver,^{7,8} were severely reduced in liver, brain, and fibroblasts of Zellweger patients.⁹⁻¹² In accordance, plasmalogen-type alkoxyether lipids were shown to be virtually absent in several tissues of Zellweger patients, and reduced in their erythrocytes and fibroblasts.^{13,14} However, the phosphatidylethanolamine (PE) plasmalogen content of erythrocytes of Zellweger patients was subsequently found to be reduced in patients less than 20 weeks of age, but normal in older Zellweger patients.¹⁵

The alkoxyether lipid platelet activating factor (PAF), 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine,^{16,17} is synthesized by several cell types, including endothelial cells,¹⁸ leukocytes,¹⁹ and platelets.^{20,21} It induces both platelet²² and leukocyte^{23,24} aggregation and degranulation, and is implicated in several pathological processes, such as inflammation,²⁴ asthma,²⁵ anaphylaxis,²⁶ hypotension,^{27,28} and

bacterial endotoxin-induced hypotension²⁹ and thrombocytopenia.³⁰ Cells and tissues with a deficient PAF synthesis would provide a useful tool to study the mechanisms involved in PAF-mediated processes in vitro.³¹ In the present study we provide evidence that the Ca²⁺-ionophore A23187-induced PAF synthesis by leukocytes of Zellweger patients may be an age-related deficiency similar to the plasmalogen content of their erythrocytes.¹⁵

MATERIALS AND METHODS

Patients. All patients demonstrated typical clinical and biochemical features of the Zellweger syndrome (Table 1). Informed consent was obtained from their parents and the study was approved by the medical ethical committee of the Academic Medical Center.

Methods. Leukocytes enriched in polymorphonuclear leukocytes (PMN) were isolated, and PAF synthesis measured as described previously.³¹ Briefly, 0.4 ml of 1×10^7 leukocytes/mL in a Tris-Tyrode buffer (137 mmol/L NaCl, 2.6 mmol/L KCl, 0.36 mmol/L NaH₂PO₄, 5.5 mmol/L glucose, 2.0 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, 10 mmol/L Tris, 0.25% albumin, pH = 7.35) were stimulated for 45 minutes with 10 μ Mol/L A23187. PAF was isolated by extraction with ethanol and chloroform, further purified by thin layer chromatography, and quantified by aggregation measurements of gel-filtered rabbit platelets.

The isolation of erythrocytes¹⁵ and determination of their plasmalogen content,¹⁴ and the measurements of DHAP-AT activity,⁹ plasmalogen content,¹⁴ percentage of particle-bound catalase,³² and de novo plasmalogen biosynthesis³³ of cultured skin fibroblasts were performed as described before.

RESULTS

The results of the analyses of cells of the Zellweger patients are presented in Table 2. The analyses of fibroblast parameters, ie, DHAP-AT activity, catalase latency test, PE plasmalogen content (with the exception of the oldest patient tested), and de novo phosphatidylcholine (PC) and PE plasmalogen synthesis, were abnormally reduced in all patients tested and thus confirmed the diagnosis of Zellweger syndrome in patients no. 1 and 3 through 7. The diagnosis in patient no. 2 was confirmed by DHAP-AT measurement of the thrombocytes (patient no. 2, 0.95; eight controls 1.97 to 3.41 nmol $\cdot$ 30 minutes⁻¹ $\cdot$ mg protein⁻¹).

PAF synthesis was undetectable in leukocytes from two patients studied at 23 and 29 days of age and reduced in a third patient studied at 62 days of age. In fact, in this third patient PAF synthesis by leukocytes collected at 58 days of age could not be demonstrated. The PAF synthesis was in the

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Table 1. Clinical and Biochemical Characteristics of the Zellweger Patients

Patient no.	1	2	3	4	5	6	7
Craniofacial dysmorphism	+	+	+	+	+	+	+
Hypotonia	+	—*	+	+	+	+	+
Tapetoretinal degeneration/cataract	nd	+	+	+	+	+	+
Hepatomegaly/liver dysfunction	+	+	+	+	+	+	+
Psychomotoric retardation	+	nd	+	+	+	+	+
Pipecolic acid in serum and/or urine	↑	↑	↑	↑	↑	↑	↑
T- and DHCA in serum and/or urine	↑	nd	↑	↑	↑	↑	↑
p-OH-phenyllactate	↑	nd	nd	nd	↑	↑	nd
Cysts in kidney	+	nd	+	nd	nd	nd	nd
Cause of death	B	U	P	SA	U	P	SA

Abbreviations: +, present; —, absent; nd, not determined; ↑, increased; SA, still alive; P, pneumonia; B, brain hemorrhage; U, unknown; T- and DHCA, tri- and dihydroxycoprostanic acid.

*Patient demonstrated hypertonica.

low normal range in four patients tested between 4 and 61 months of age. The three patients with the abnormal PAF synthesis died before 3 months of age, and the other patients were still alive after 32 months.

The patients with abnormal PAF synthesis also showed a significantly reduced PE plasmalogen content of the erythrocytes, whereas this parameter was virtually normal in the other patients.

DISCUSSION

The first steps of the de novo alkoxyether lipid biosynthesis, which are dependent on DHAP-AT and alkyl-DHAP synthase, are localized in the peroxisome.^{7,8} Subsequently, in several extra-peroxisomal steps 1-0-alkyl-2-acyl-glycerol-3-phosphocholine and 1-0-alkyl-2-acyl-glycerol-3-phosphoethanolamine are formed.⁸ These molecules may finally be oxidized to the 1-0-alkenyl compounds, ie, plasmalogens. However, 1-0-alkyl-2-acyl-glycerol-3-phosphocholine may also be stored in the cellular membrane and, upon cell stimulation, converted into the 2-acetyl compound, PAF.³⁴

The impairment of de novo alkoxyether lipid synthesis in Zellweger patients is clearly established, ie, DHAP-AT and alkyl-DHAP synthase activities as well as plasmalogen content and de novo plasmalogen synthesis³³ are reduced or

virtually absent in several tissues and cells of these patients.⁹⁻¹⁴ However, evidence was put forward that the plasmalogen content of erythrocytes of these patients reaches normal levels if blood is collected after 3 to 5 months of age.¹⁵ In the present study we have provided evidence that deficient PAF synthesis by the leukocytes of Zellweger patients may also be an age-related phenomenon limited to the first 3 months of life. Several explanations for these findings are possible. First, with age the patients may partly overcome the deficient alkoxyether lipid synthesis. Plasmalogens, DHAP-AT, and alkyl-DHAP synthase activities are never completely absent in tissues of Zellweger patients,⁹⁻¹⁴ and a residual capacity for de novo plasmalogen biosynthesis is clearly present in their cultured skin fibroblasts.³³ These residual enzyme activities may be due either to the presence of a few microperoxisomal structures,³⁵ or to a reduced activity of peroxisomal enzymes that are synthesized on free polyribosomes and remain in the cytosol instead of being incorporated in pre-existing peroxisomes.^{36,37} A possible mechanism for the presumed improvement in PAF synthesis and erythrocyte plasmalogen content might be the positive selection with age of progenitor cells in the bone marrow with slightly more residual alkoxyether lipid synthesis capacity. A second possible explanation for the age-related PAF synthe-

Table 2. Alkoxyether Lipid Parameters in the Zellweger Patients

Patients	1	2	3	4	5	6	7	Age Controls	Adult Controls
Age at sampling	23 d	29 d	62 d	4 mo	40 mo	54 mo	61 mo	<3 mo	—
Age at death	50 d	33 d	91 d	>38 mo	>60 mo	56 mo	>94 mo	—	—
PAF synthesis*	<2	<3	110	731	232	273	572	249-2757 (n = 4)	291-5433 (n = 5)
Erythrocyte PLG†	23.8‡	21.0	4.8	41.8	34.7	57.3	41.0	37.6-47.5 (n = 9)	36.9-53.1 (n = 66)
Fibroblast									
DHAP-AT§	0.38	—	0.81	0.97	0.34	0.88	0.48	—	5.4-19.8 (n = 27)
Catalase latency	<5	—	<5	<5	<5	<5	—	—	50-81 (n = 9)
PLG†	26.4	—	21.7	34.4	31.6	26.1	48.6	—	46.4-62.4 (n = 13)
De novo synthesis A¶	2.2	—	0.8	1.2	1.4	2.9	1.7	—	13.6-30.4 (n = 19)
De novo synthesis B¶	57.9	—	26.9	49.5	40.4	72.1	55.0	—	84.3-93.4 (n = 19)

*PAF synthesis is expressed as picomoles PAF/10⁸ PMN.

†The plasmalogen content (PLG) is expressed as the percent plasmalogen of PE relative to the total PE.

‡Blood for erythrocyte PLG collected at 42 days of age.

§Fibroblast DHAP-AT activity is expressed as nanomoles · 2 h⁻¹ · mg protein⁻¹.

||Catalase latency of fibroblasts is expressed as the percent particle-bound catalase.

¶De novo plasmalogen synthesis of the fibroblasts is expressed as the percent [¹⁴C]-hexadecanol uptake in plasmalogen of either PC relative to the incorporation in the total PC (A), or PE relative to the incorporation in the total PE (B).

sis deficiency would be the cellular uptake of alkoxyether lipids or precursors from the diet, but this possibility is less likely as the plasmalogen content of erythrocytes of patients suffering from the rhizomelic type of chondrodysplasia punctata remains severely reduced even up to 16 years of age.³⁸ However, a third explanation cannot be excluded: Possibly the PAF synthesis in the patients tested at 4 months to 5 years of age has also been normal earlier in life. A PAF synthesis deficiency would then be present only in the more severely affected patients who die within a few months of age. Unfortunately, this possibility could not be tested as the older patients were not investigated at an early age, and the patients with the deficient PAF synthesis died before 3 months of age.

PAF has been ascribed an important role in inflammation.²⁴ Interestingly, Zellweger patients frequently suffer from pneumonia and it is a frequent cause of their death.¹ Of

course, severe neurological impairment, malfunction of several organs, and possibly the reduced cellular plasmalogen content may cause the frequent pneumonia, but it is tempting to speculate that the deficient PAF synthesis may also be a pathogenetic factor.

In summary, we conclude that the deficiency of PAF synthesis by leukocytes of Zellweger patients may be an age-related phenomenon, and this deficiency may be of etiological importance for some of the complications in these patients. However, a relation of the deficient PAF synthesis and the severity of the disease cannot be excluded.

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Simian Virus 40-Transformed Adherent Cells From Human Long-term Marrow Cultures: Cloned Cell Lines Produce Cells With Stromal and Hematopoietic Characteristics

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Adherent cells from long-term marrow cultures from 23 individuals were transformed with wild-type simian virus 40 (SV40). After transformation, cloned cell lines were developed that even after rigorous subcloning invariably produced both stromal cells and round cells. The stromal cells expressed cytoskeletal filaments similar to those of long-term marrow culture adherent cells and produced interstitial and basal lamina collagen types. The round cells had the electron microscopic appearance of primitive hematopoietic cells and when examined with cytochemical stains and monoclonal antibodies to hematopoietic differentiation antigens had reaction patterns suggestive of cells from several lineages. Most round cells expressed the

pan-hematopoietic T-200 determinant, and lesser percentages expressed the early T cell antigens CD-1 and CD-3, HLA-DR determinants, the monocytic antigen recognized by Leu M3, and the myeloid antigens detected by monoclonal antibodies 1G10 and 12.8. In addition, when plated in semisolid medium in the presence of a source of colony-stimulating activity, up to 11% of the cells formed colonies consisting of blastlike cells that also expressed hematopoietic cell surface determinants. The data suggest that adherent cells in long-term marrow cultures contain a cell that after transformation by SV40 obligately produces cells with hematopoietic as well as stromalike features.

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THE HUMAN LONG-TERM MARROW culture (LTMC) system permits the proliferation of hematopoietic progenitors for several months in the presence of a layer of adherent or stromal cells that act as an *in vitro* microenvironment.¹⁻³ The adherent layer is heterogeneous in its cellular content and contains variable numbers of fat-containing cells and macrophages, a minor population of round hematopoietic cells, and a major population of flat angulated cells of fibroblastic appearance. Nonhematopoietic properties of stromal cells include the synthesis of a complex extracellular matrix similar to that synthesized by cultured smooth muscle cells.⁴⁻⁶ Many cells in the adherent layer bear functional receptors for platelet-derived growth factor⁷ and express actin filaments recognized by a monoclonal antibody (CGA-7) that reacts with an actin epitope otherwise present only on smooth muscle cells.⁸

Attempts to grow clones of human stromal cells similar to

those developed from murine stromal cells have had limited success.⁹⁻¹⁴ In the present study, we used wild-type simian virus 40 (SV40) to transform cells and generate stromal cell lines on 25 marrow samples from 23 individuals. Seven of the resultant cell lines, including three from females heterozygous for the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD), were cloned at limiting dilutions. All clones produced both adherent cells with stromal characteristics and round, loosely adherent cells with morphological and phenotypic characteristics of hematopoietic cells of several lineages. Moreover, when transformed cells were plated in semisolid medium in the presence of a source of colony-stimulating activity (CSA), colonies of blastlike cells were formed that expressed hematopoietic determinants.

MATERIALS AND METHODS

Subjects. LTMC were generated from clinical marrow aspirates obtained after informed consent from subjects with the diagnoses listed in Table 1 under protocols approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. Three patients with acute lymphoblastic leukemia were heterozygous for G6PD.

Cell cultures. LTMC were grown as described.^{2,4} Confluent adherent layers from 4-week-old cultures were treated with trypsin (1:250; Difco, Detroit) in EDTA and passaged into fresh flasks. SV40 (SV40/VERO 7; Meloy Laboratories, Springfield, VA) was added (ten particles per cell), and after 12 hours at 37°C, the adherent cells were harvested and reseeded in microtiter plates (Costar, Cambridge, MA) at 1,000 cells/well in medium supplemented with 30% conditioned medium from the KG-1 cell line.¹⁵ The KG-1 cell line produces a factor that stimulates proliferation of cultured, passaged adherent cells from LTMC (unpublished data). Between 50% and 95% of the wells gave rise to passagable cell lines. After two to three passages, the KG-1 conditioned medium, the horse serum, and hydrocortisone were deleted, and the concentration of fetal calf serum in the medium was gradually decreased to 5%. Some cell lines were later adapted to serum-free conditions in HL-1 medium (Ventrex; Portland, ME). The absence of serum did not alter the morphology of the cells.

Clonal growth. Limiting dilution cloning was performed on cell lines from seven of the patients including the three who were heterozygous for G6PD. After treatment of the cell lines with trypsin, a single-cell suspension was obtained by vigorously passing

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Table 1. SV-40-Transformed Stromal Cell Lines

Cell Line Number	Patient Diagnosis	Longevity (mo)	Number of Passages
1	Normal donor	8	22
2	Normal donor	8	21
3	Normal donor	5 +	16 +
4	CML	7	26
5	CML blast crisis	9	27
6	CML	2 +	7 +
7	CML	2 +	10 +
8	CML	2 +	10 +
9	CML	6	32
10	ALL remission*	9	28
10A†		7 +	21 +
11	ALL remission*	11	36
12	ALL remission	6	20
13	ALL remission*	8	24
14	ALL relapse*	6	18
15	AML remission	5	13
16	AML at diagnosis	4 +	15 +
17	Aplastic anemia	7	23
17A‡	After transplant	3	10
18	Aplastic anemia	6	23
18A‡	After transplant	5 +	21 +
	Lymphoma after trans-		
19	plant	4 +	12 +
20	CML after transplant	7	28
21	CML after transplant	12 +	40 +
	Preleukemia after trans-		
22	plant	6	20
23	CML after transplant	6 +	20 +

Abbreviations: CML, chronic myelogenous leukemia; ALL, acute lymphocyte leukemia.

*The patient was heterozygous for G6PD.

†Transformation of a second marrow sample obtained 8 months after the first.

‡Second sample obtained after bone marrow transplantation.

the cells through a 26-gauge needle. The trypan blue dye-excluding cells were then counted, and microtiter dishes were seeded in a limiting dilution assay at calculated cell concentrations of 0.5 to 100 cells/well. The plates were evaluated with an inverted microscope, and wells with growth were serially passaged until sufficient cells were available for characterization studies. Only wells with a single focus of growth were considered to contain potential clones. G6PD in the putative clones from the three G6PD heterozygous patients was assayed by starch gel electrophoresis.¹⁶ To estimate the relative amounts of G6PD activity in round cells and stromalike cells in these cultures, loosely adherent cells and nonadherent cells were harvested by gentle agitation. This population contained predominantly round cells that bore hematopoietic determinants. Adherent cell layers with most of the round cells removed by agitation were separately harvested from a culture of the opposite G6PD type from the round cells. A series of mixed samples was then prepared that contained between 5% round cells and 95% stromal cells or between 5% stromal cells and 95% round cells and tested for G6PD activity by starch gel electrophoresis.

Characterization with monoclonal antibodies. For characterization with monoclonal antibodies, cells from both native and subcloned cell lines were passaged into chamber slides (Lab-tek; Miles Scientific, Naperville, IL), and when confluent, the cells were fixed with absolute methanol for examination with monoclonal antibodies to the "T" translational product of SV40¹⁷ (Oncogene Sciences, Mineola, NY) and to cytoskeletal proteins or in 2%

formaldehyde and 0.1% glutaraldehyde (4°C; 30 minutes) for assays with antibodies to cell surface epitopes. The cells were then exposed to an appropriate dilution of the test antibody or an irrelevant isotype control antibody for 30 minutes at room temperature. After washing, a goat fluorescein isothiocyanate-conjugated, F(ab')₂ antimouse IgG or IgM (Cappel Laboratories, West Chester, PA) was applied for 30 minutes before examination under a fluorescence microscope. Test antibodies included the anticytoskeleton antibody CGA-7, which recognizes smooth muscle actin; HHF, which reacts with actin in smooth, skeletal, and cardiac muscle; and 43βE8, which reacts with vimentin in mesenchymal cells but not in hematopoietic cells (kindly donated by A. Gown).^{8,18} The monoclonal antibodies to hematopoietic determinants included 9.4, which recognizes the T-200 common hematopoietic determinant^{19,20}; 7.2, which reacts with an HLA-DR framework determinant; 64.1, which recognizes CD-3; 35.1, which reacts with CD-2 (all kindly donated by Paul Martin)^{21,22}; anti-Leu 6,²³ an antibody to the CD-1 determinant of immature thymocytes; Leu M3, an antibody that reacts with nearly all monocytic cells²⁴ (Becton Dickinson, Mountainview, CA); 12.8 and 1G10, which react with early and late myeloid cells, respectively^{25,26} (kindly donated by Irwin Bernstein); and 60.1 and 60.3, which recognize the α and β subunits of the C3Bi receptor complex, respectively^{27,28} (kindly donated by Patrick Beatty).

Cytochemical staining and rosetting with aminoethylthiuronium (AET)-treated sheep RBC were performed by conventional techniques on trypsin-harvested cells.^{29,30} For electron microscopy, cultures were fixed in one-half-strength Karnovsky's fixative for one hour at room temperature and processed routinely for scanning and transmission electron microscopy as described.⁶

Colony formation. Trypsinized cells from the cell lines were washed and seeded at between 1,000 and 25,000 cells/mL in 35 × 10-mm tissue culture dishes (Lux Plastics; Miles Scientific) and medium consisting of 20% fetal calf serum and 1.0% methylcellulose in Iscove's medium containing 10⁻⁶ mol/L 2-mercaptoethanol and varying concentrations of phytohemagglutinin-stimulated lymphocyte conditioned medium (PHA-LCM) or recombinant GM-CSF as a semipurified COS cell supernatant (kindly donated by K. Kaushansky). The culture dishes were examined serially with an inverted microscope, and colonies consisting of >50 cells and clusters of >20 cells were counted after 14 days. Only cell aggregates consisting entirely of round cells were enumerated. Individual colonies were then plucked, pooled, and characterized as described earlier.

Biochemical studies. Tests to confirm the identity of cytoskeletal proteins using the Western technique were performed by exposing cells harvested in EDTA and benzamidine (10 mmol/L) to sonication and then preparing a suspension in sodium dodecyl sulfate (SDS) with β-mercaptoethanol. The samples were run on 8% polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose and incubated with monoclonal antibodies, and the patterns were developed by using glucose oxidase-conjugated goat antimouse antibody.¹⁸

The presence of collagenous proteins and thrombospondin was tested by labeling stromal cells with ³⁵S-methionine and extracting them in SDS before electrophoresis on 7.5% polyacrylamide gels. The antithrombospondin antibody was prepared by immunizing rabbits against purified thrombospondin from human platelets as described.^{31,32} An IgG fraction was obtained by repeated precipitation with ammonium sulfate. Contaminating antifibrinogen antibody activity was removed by affinity chromatography using Sepharose 4B linked to human fibrinogen. The resulting preparation bound to thrombospondin but not to fibronectin, fibrinogen, and human collagen types I, III, IV, and V.³³ The anti-type IV collagen antiserum IgG fraction was similarly prepared. It reacted with type IV collagen but not with types I and III and only slightly with type V collagen. For radiolabeling, washed cells were cultured for 18 hours

in methionine-deficient medium. Immunoprecipitations were performed as described³⁴ and analyzed by autoradiography after gel electrophoresis.

Platelet glycoprotein 1b (Gp1b) was measured by placing cell lysates in wells with adsorbed rabbit polyclonal anti-Gp1b affinity-purified antibody in the presence of EDTA, *N*-ethyl maleimide, and leupeptin.³⁵ A mouse monoclonal anti-Gp1b (C7E10, kindly donated by Diane Nugent) was added after washing and was followed by alkaline phosphatase-labeled goat antimouse antibody (Cappel) and phosphatase substrate. The amount of Gp1b present was estimated by using a standard of purified human platelet glycolalicin. The reactivity of each of the other antibodies was similarly confirmed by using an enzyme-linked immunosorbent assay (ELISA).^{36,37}

RESULTS

Initially, the cell lines consisted predominantly of spindle-shaped and flat-angulated cell similar to those observed in nontransformed adherent cell layers from LTMC. Increasing numbers of round cells, some of which were loosely adherent, appeared after six to nine passages (Fig 1A). Scanning electron microscopy of the cultures revealed small, round cells adhering to flat, stromal cells (Fig 1B). Transmission electron microscopy of these same cultures demonstrated the presence of undifferentiated hematopoietic-appearing cells admixed with an adherent cell population that appeared similar to stromal cells found in untransformed LTMC (Fig 1C).

Cloning studies. Seven of the cell lines were cloned at limiting dilutions between passages 6 and 13 (Table 2). Most wells seeded with fewer than ten cells had only a single focus of growth when examined at 1 week. Wells with growth were serially passaged three to four times until sufficient numbers of cells were available for characterization. The linearity of the semilogarithmic plots of the percentage of wells without growth v the number of cells plated allowed calculation of the cloning efficiency of the lines.³⁸ This varied between 5% and 50% as shown in Table 2. G6PD was used as a cell marker to determine whether the putative clones originated from single cells. Limiting dilution cloning was performed on cell lines from the three G6PD heterozygous patients at times when the native cell lines expressed both A- and B-type G6PD. All but one of the 68 cell lines initiated with ten or fewer cells per well expressed only either A- or B-type G6PD, further attesting to their clonal nature (Table 3). Each of the cell lines that manifested only a single G6PD enzyme appeared morphologically identical to the parent line and consisted of both round cells and stromal-like cells. When G6PD, type A, round cells were mixed in various proportions with G6PD, type B, stromal cells and the relative activities were determined, the amount of G6PD activity per cell was approximately equal. Five percent G6PD A-type round cells were detectable when mixed with 95% type-B stromal cells, and conversely, 5% added type-B stromal cells were detectable when mixed with 95% type-A round cells. Since the proportion of round cells in the tested cloned cell lines was at least 30%, these data suggest that they should have been detectable by G6PD if they had been of the opposite enzyme type. Therefore, the G6PD data suggest that these putative clones were true clones.

Cell line characterization. Subsequent characterization

studies were performed on both subcloned cell lines initiated with fewer than ten cells per well from the limiting dilution cultures and the native cell lines. Most of the cell lines grew rapidly for 20 to 30 passages, with a doubling time of three to six days, and then entered a crisis. Only one line was rescued after entering crisis (line 21). This cell line has been maintained in continuous culture for more than 48 passages (over 16 months). All characterization studies were performed with cell lines between the tenth and 20th passages. Examination of the cell lines by indirect immunofluorescence with a monoclonal antibody to the large T antigen of SV40 demonstrated the presence of the antigen in nearly all nuclei of cells

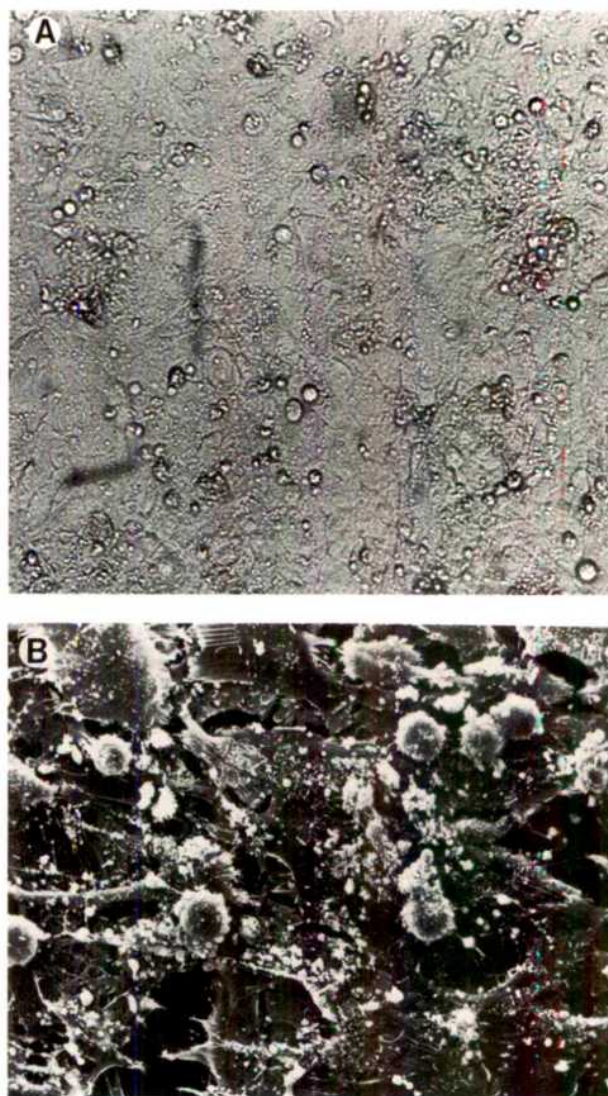


Fig 1. (A) Phase-contrast photomicrograph of subcloned transformed stromal cell line 10. Note the confluent adherent cell layer and the loosely adherent round cells. (B) Scanning electron microscopic appearance of transformed subcloned stromal cell lines. (Original magnification $\times 320$; current magnification $\times 176$.) A number of round cells can be seen associated with adherent stromal cells. (C) Transmission electron micrographs of the same cultures. (Original magnification $\times 13,000$; current magnification $\times 9,880$.) Round cells are present above the adherent stromal cells (s). s refers to the location of the bottom of the culture flask.

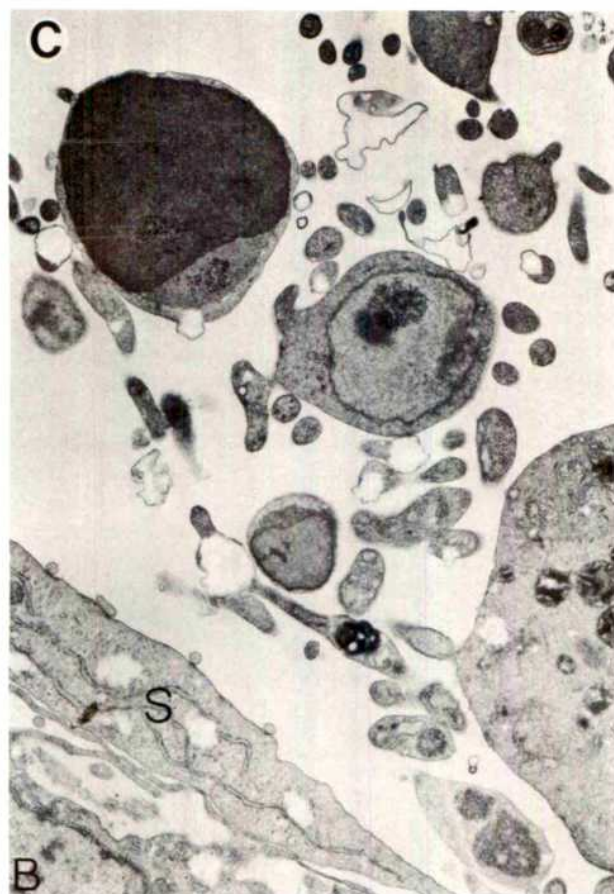


Fig 1. (Cont'd).

from native and subcloned lines (data not shown).¹⁷ Southern blotting hybridization analysis of DNA from cell lines 3 and 5 demonstrated integration of SV40 DNA (data not shown).

Stromalike cells. The cell lines were examined with monoclonal antibodies recognizing cytoskeletal filaments found in stromal cells from LTMC.⁸ Reactivity of round cells was rarely seen. In contrast, nearly all elongated, adherent cells expressed actin and vimentin as recognized by the antimyosin actin antibodies CGA-7 and HHF and the antivimentin antibody 43βE8.^{8,18} Unlike the filamentous actin network found in untransformed stromal cells from LTMC,⁸ the SV40-transformed cells displayed diffuse cytoplasmic fluorescence. Even the vimentin network was not as well delineated as in nontransformed cells. However, Western

Table 2. Limiting Dilution Cloning

Cell Line	No. Cells/Microtiter Well (0.2 mL)					Cloning Efficiency (%)
	0.5	1.0	2.0	5.0	10.0	
1	7/45*	22/88	51/96	84/86	ND	33
10	ND	7/100	ND	23/100	48/100	7
10 (reclone)	21/144	20/144	ND	44/144	ND	14
11	18/138	63/288	63/96	ND	ND	50
12	4/96	15/96	23/96	54/96	ND	16

Abbreviation: ND, not determined.

*The numerator is the number of wells with passageable cell lines. The denominator is the number of wells seeded.

Table 3. Limiting Dilution Cloning of Cell Lines From Three G6PD Heterozygous Patients

Cell Line	Passage Cloned	G6PD A:B	No. Cells/Well	No. Wells With G6PD		
				A	B	A + B
10	6	1:1	1	3	1	0
			5	12	2	1
			10	11	2	0
			100	17	0	7
14	13	4:1	10	0	1	0
			25	1	1	0
			50	1	0	0
			100	0	1	0
13	11	1:1	0.5	7	2	0
			1	9	0	0
			2	11	2	0

blot analysis confirmed the presence of both actin and vimentin in the transformed cells (Fig 2A). These data suggest that, as in other SV40-transformed cell lines, actin did not polymerize normally in the transformed stromal cells.³⁹ Analysis of collagenous proteins synthesized by the transformed cell lines demonstrated the presence of basal lamina collagen (type IV), a collagen synthesized by both smooth muscle and by LTMC adherent cells but not by marrow fibroblasts^{4,5} (Fig 2B).

Round cells. A series of monoclonal antibodies recognizing myeloid and lymphoid differentiation antigens was also used to characterize the cells from native and subcloned cell lines. Only the round cells and not the elongated stromalike cells reacted with these antibodies (Table 4). In indirect immunofluorescence assays, 55% ± 4% (mean ± SEM) of the round cells reacted with a monoclonal antibody with a specificity for the common leukocyte T-200 antigen (9.4) (Fig 3A and B)¹⁸; 15% ± 8% reacted with antibody Leu 6, which recognizes the CD-1 determinant on immature thymocytes²³; 22% ± 5% reacted with antibody 64.1, which recognizes the T cell receptor complex (Tp19-29, CD-3) (Fig 3C and D)²¹; and lower frequencies of cells reacted with antibodies recognizing CD-2, the sheep RBC receptor (35.1),²¹ the monocyte-macrophage antigen identified by Leu M3,²⁴ and the myeloid antigens recognized by antibodies 12.8 (Fig 3E and F) and IG10.^{25,26} In addition, 15% ± 7% of the round cells expressed HLA-DR framework determinants (antibody 7.2). Approximately 3% of the round cells reacted with antibody 60.3, which recognizes the β subunit of the C3Bi receptor, an antigen expressed by lymphoid and myeloid cells.^{27,28} The cells did not react with antibodies recognizing the α subunit of the C3Bi complex (60.1), but small numbers of round cells reacted with both a monoclonal antibody and an affinity-purified polyclonal antibody to platelet Gp1b.³⁵ By using an enzyme-linked immunosorbent assay, the cellular content of GPIb was estimated to be 13, 23, and 2 ng/mg protein in subcloned cell lines 7, 2, and 19, respectively. The assay is capable of detecting <1 ng of glyocalicin/mg of protein (unpublished data).

Analysis of five subclones from different patients by using cytochemical stains showed that most cells contained acid phosphatase,²⁹ some cells contained large amounts of PAS-positive material,²⁹ and between 5% and 42% reacted with

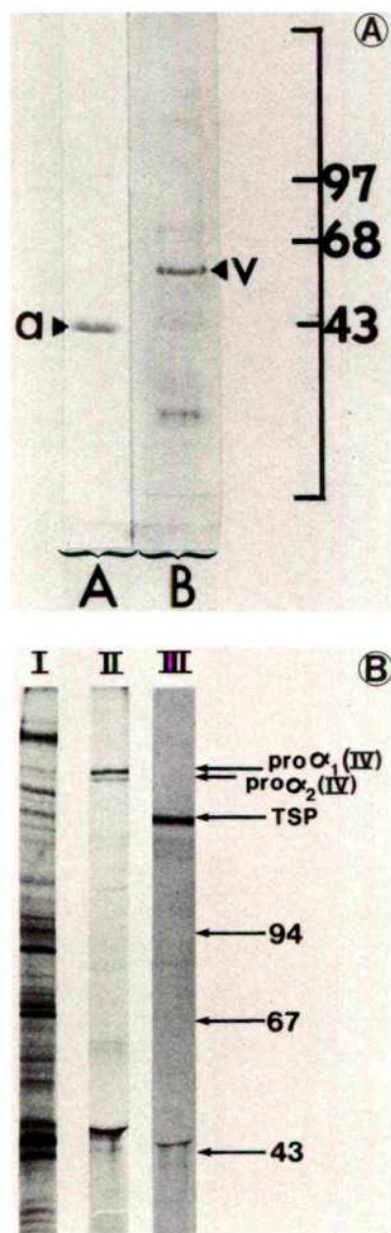


Fig 2. (A) Western blots of transferred cell extracts with antiactin (A) and antivimentin (v) monoclonal antibodies. (B) Immunoprecipitated bands from SDS-polyacrilamide (7.5%) gels of [^{35} S] methionine-labeled, transformed stromal cells. Lane I, whole medium; lane II, radioimmune precipitation with anti-type IV collagen; lane III, radioimmune precipitation with antithrombospondin. pro α_1 (IV) and pro α_2 (IV) refer to type IV procollagen chains; Tsp refers to thrombospondin; the numbers 94, 67, and 43 indicate the positions of the molecular weight standards (phosphorylase B, albumin, and ovalbumin [Pharmacia Fine Chemicals, Piscataway, NJ], respectively).

nonspecific esterase using α -naphthyl butyrate as a substrate.²⁹ Incubation of line I with phorbol myristate acetate (PMA) at 10^{-7} mol/L for four days increased the frequency of nonspecific esterase-positive cells from $7\% \pm 3\%$ (mean $\pm$ SD) of cells to $24\% \pm 6\%$. Neither peroxidase nor chloroacetate esterase was detectable in any of the cell lines.²⁹ Between 3% and 25% of trypsin-harvested cells formed rosettes with

AET-treated sheep RBC when subclones from patients 2, 4, and 5 were tested.

Colony formation. To determine whether cells in the SV40-transformed adherent layers formed colonies in semisolid medium, the cultures were treated with trypsin, and single-cell suspensions were prepared and plated in semisolid medium with and without PHA-LCM or recombinant GM-CSF. No evidence of cell division was seen until day 5 when doublets were first noted. Thereafter growth was rapid, and colonies of up to 1,000 cells formed by day 14 (Fig 4A). Each of five tested cell lines formed colonies. Only a single type of colony was observed. It consisted of a large, tight ball with occasional loose cells around its periphery. Colonies grew only in the presence of a source of CSA. There was a dose-response relationship with PHA-LCM (Table 5). As with CFU-GM, optimal colony formation was observed when 20% PHA-LCM was added (Fig 5A). The number of colonies grown was linearly related to the number of cells plated (Fig 5B). In the presence of 20% PHA-LCM, 1.1%, 2.3%, and 11.0% of the cells seeded formed colonies in lines 10A, 11, and 9 respectively. When tested with recombinant GM-CSF, cluster formation (defined as round cell aggregates of between 20 and 50 cells) was observed at concentrations as low as 10 U/mL; some large colonies were present in plates containing 1,000 and 10,000 U/mL (Table 6). However, even if the numbers of colonies and clusters that grew with 10,000 units of GM-CSF are combined, the cloning efficiency was only 23% of that observed with 20% PHA-LCM.

When colonies were individually plucked and placed in suspension culture, some cells became adherent and assumed a stromalike configuration. Cell division continued, and within 2 weeks, these suspension cultures reassumed the appearance of the native, SV40-transformed cell lines in suspension culture. When the colonies were plucked, disaggregated with trypsin to obtain single-cell suspensions, and then reseeded in semisolid medium with PHA-LCM, secondary colonies were formed with a reseeding efficiency of 1.1% with 20% PHA-LCM and 0.5% with GM-CSF (10,000 U/mL).

Characterization of colonies. Most cells in the colonies appeared to be undifferentiated, but occasional cells had monocytoid or lymphocytoid features (Fig 4B). The majority of cells in the colonies reacted with nonspecific esterase stains, and most ingested latex beads. The cells did not react with peroxidase. Indirect immunofluorescence assays with monoclonal antibodies on colonies from three different cell lines demonstrated that $50\% \pm 5\%$ of the cells reacted with 9.4, an antibody to the common hematopoietic T-200 determinant; $39\% \pm 4\%$ reacted with 64.1, an antibody that reacts with the CD-3 complex; $12\% \pm 4\%$ reacted with antibody Leu 6, which recognizes the CD-1 determinant; $12\% \pm 2\%$ reacted with antibody 12.8, a 115-kd cell surface complex present on immature hematopoietic cells; and $18\% \pm 4\%$ reacted with 7.2, an antibody to an HLA-DR framework determinant (Fig 4C). Although the native cell lines from which the colonies were grown expressed actin and vimentin as detected by antibodies CGA-7 and 43 β E8, the cells in the colonies, like the round cells in native cultures, did not react

Table 4. Expression of Cell Surface Antigens on Cloned, SV40-Transformed Stromal Cells

Antibody Reactivity	Antigen	Cell Lineages	References	Percentages of Round Cells
9.4 (n = 25)†	T-200	Nucleated hematopoietic cells	15, 16	55 ± 4*
Leu 6 (n = 6)	CD-1	Immature thymocytes	46	15 ± 7
64.1 (n = 25)	CD-3	T cell receptor complex	22, 23	22 ± 5
35.1, 9.6 (n = 4)	CD-2	T cells, sheep RBC receptor	22, 27	6 ± 2
Leu M3 (n = 4)		Monocytes	26	7 ± 1
12.8 (n = 12)	115 kd	Immature myeloid cells	25	10 ± 2
1G10 (n = 15)	Le ^x	Granulocytes-monocytes	24	4 ± 1
7.2 (n = 4)	HLA-DR	Multiple lineages		15 ± 5
60.3 (n = 9)	β subunit, C3Bi complex	Multiple lineages	26, 27	3 ± 1
60.1 (n = 6)	α subunit of C3Bi complex	Granulocytes	27	0
C7E10 (n = 4)	Gp1b	Megakaryocytes	34	8 ± 4

Abbreviation: kd, kilodalton.

*Mean (± SEM) percentage of brightly fluorescent round cells on subcloned lines from multiple different subjects tested between the tenth and 20th passages.

†Cell lines derived from each of the 23 subjects were tested for expression of T-200 and CD-2. Expression of the other antigens was assessed on cell lines from between four and 15 of the subjects. Only minor differences in expression of these antigens were found between cell lines derived from different individuals. Not shown in the table are the results of analysis of subcloned cell lines from the limiting dilution clones from Tables 1 and 2. In each case, round cell antigen expression was nearly identical to that of the cell lines before cloning.

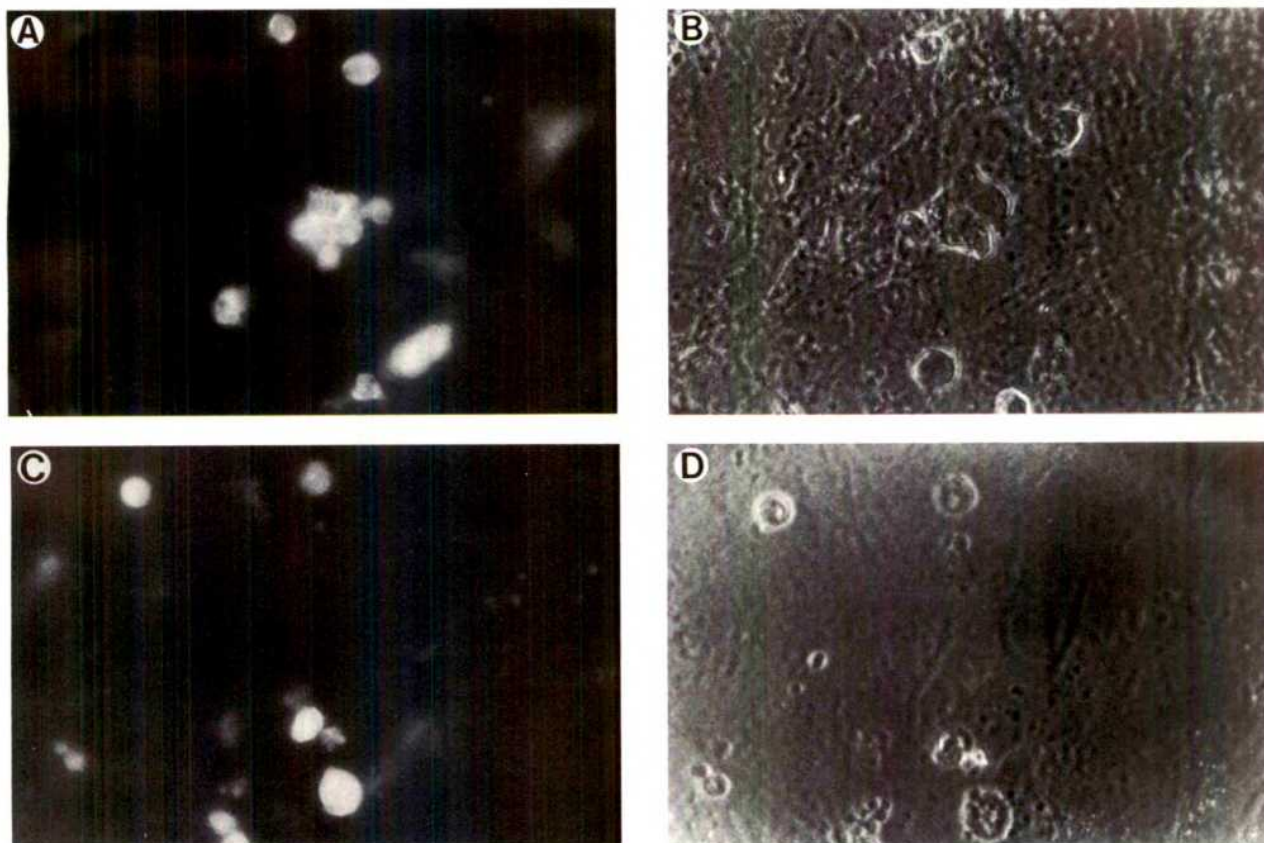


Fig 3. Immunofluorescence of transformed, subcloned cell lines. (A) Anti-T-200 or common leukocyte antigen with antibody 9.4. (C) Anti-CD3, the T cell receptor complex with antibody 64.1. (E) Anti-early myeloid antigen with antibody 12.8. (B, D, and F) Corresponding phase-contrast photomicrographs.

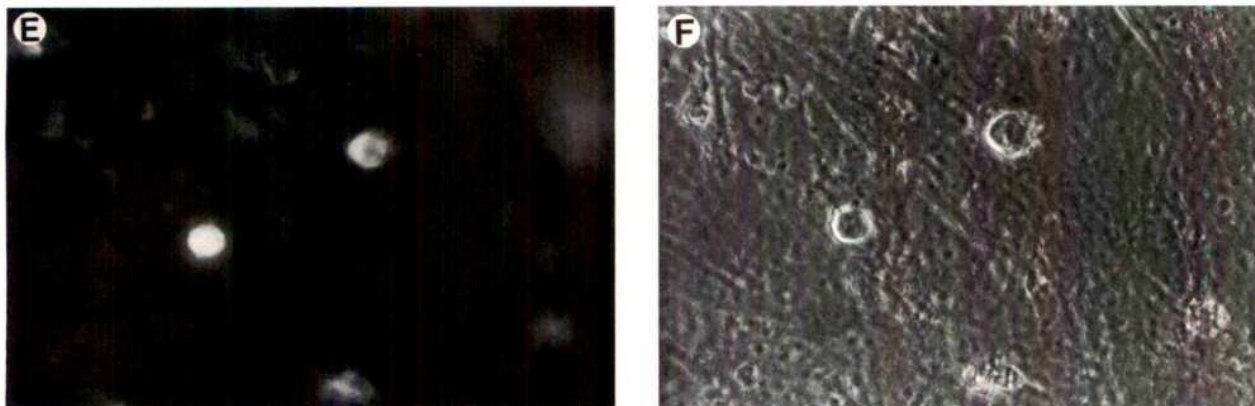


Fig 3. (Cont'd).

with these anticytoskeletal filament antibodies. However, when colonies were plucked from the semisolid medium, placed in suspension culture, and allowed to readhere for seven days, the adherent cells reexpressed actin and vimentin filaments.

DISCUSSION

Twenty-five cell lines from SV40-transformed LTMC adherent cells from 23 patients each contained cells that by morphological, cytochemical, and antigenic criteria were from disparate cell lineages. Each line contained adherent cells with morphological and biosynthetic properties similar to those of nontransformed stromal cells. These cells synthesized proteins characteristic of marrow stromal cells such as interstitial and basal lamina collagens, muscle-type actin, vimentin, and thrombospondin. Moreover, even after rigorous limiting dilution cloning, cell lines, including those shown by G6PD analysis to originate from single cells, produced loosely adherent round cells that expressed cell surface and enzymatic determinants usually associated only with hematopoietic cells. The round cells were unlikely to be simply stromal cells in mitosis for several reasons. Morphologically by both light and electron microscopy, the round cells were rarely observed in mitosis. Moreover, as the cultures became confluent and cell division slowed, the frequency of round cells increased rather than decreased. Last, when fluorescence-activated cell sorter analyses were done on trypsinized cell lines, the cells that expressed hematopoietic determinants such as CD-1, CD-3, and Leu M3 (data not shown) were among the cells with the lowest forward light scattering properties whereas the T-200 was expressed on both large and small cells.

The hematopoietic features of the round cells included expression of the common leukocyte antigen T-200 and other antigens associated with immature thymocytes such as CD-1 and CD-3. Differentiation markers of several other lineages were also detected. For example, some round cells expressed nonspecific esterase, were phagocytic for latex particles (data not shown), and expressed the antigen recognized by Leu M3 and the β subunit of the C3Bi receptor, which suggests a relation to monocytes. Platelet Gp1b was also detectable, which indicates that determinants associated

with megakaryopoiesis were expressed by some cells. The increase in expression of nonspecific esterase activity observed after the addition of PMA suggested that differentiation of these cell lines could be further influenced. Surprisingly, despite their derivation from normal donors, patients with aplastic anemia, and patients with acute and chronic leukemias, the expression of cell surface antigens on all cell lines was similar. Each expressed CD-1, CD-3 and T-200 with similar frequencies on round cells. However, the expression of antigens recognized by antibodies to myeloid differentiation antigens and to platelet Gp1b was more variable. These observations suggest that SV40 transforms a primitive cell not involved by these disease processes.

Although each of the several hundred cell lines grown from these patients produced cells that were both stromalike and round, without using genetic markers, the possibility could not be excluded that each cell line arose from two or more intimately associated cells of different lineages. For this reason, studies were done of limiting dilution cloning of SV40-transformed stromal cells from patients with cellular mosaicism for G6PD. The G6PD system has been used to demonstrate the clonal origin of hematopoietic colonies.^{40,41} When cell lines from three G6PD heterozygous patients were cloned at limiting dilutions, all but one of 64 cell lines originating at ten or fewer cells per well expressed either A or B G6PD, thereby indicating their single-cell origin. When lines originating at 100 cells/well from cell line 10 were tested, seven of 24 expressed both A and B G6PD, thereby indicating derivation from several cells. The sensitivity of the G6PD system is such that the addition of 5% of cells of the opposite enzyme type can be detected. Approximately 30% of cells in the transformed cultures are round, and the remainder appear stromal. The G6PD activity per cell of round and stromal cells from the transformed cultures is approximately equivalent. These data suggest that subcloned cell lines derived from wells seeded with fewer than ten cells are probably clones.

Two G6PD type B clones from cell line 10, one type A and one type B clone from cell line 14, and two type B clones from cell line 13 were characterized by immunofluorescence with monoclonal antibodies and were found to express muscle-type actin with antibodies CGA-7 and HHF in the stroma-

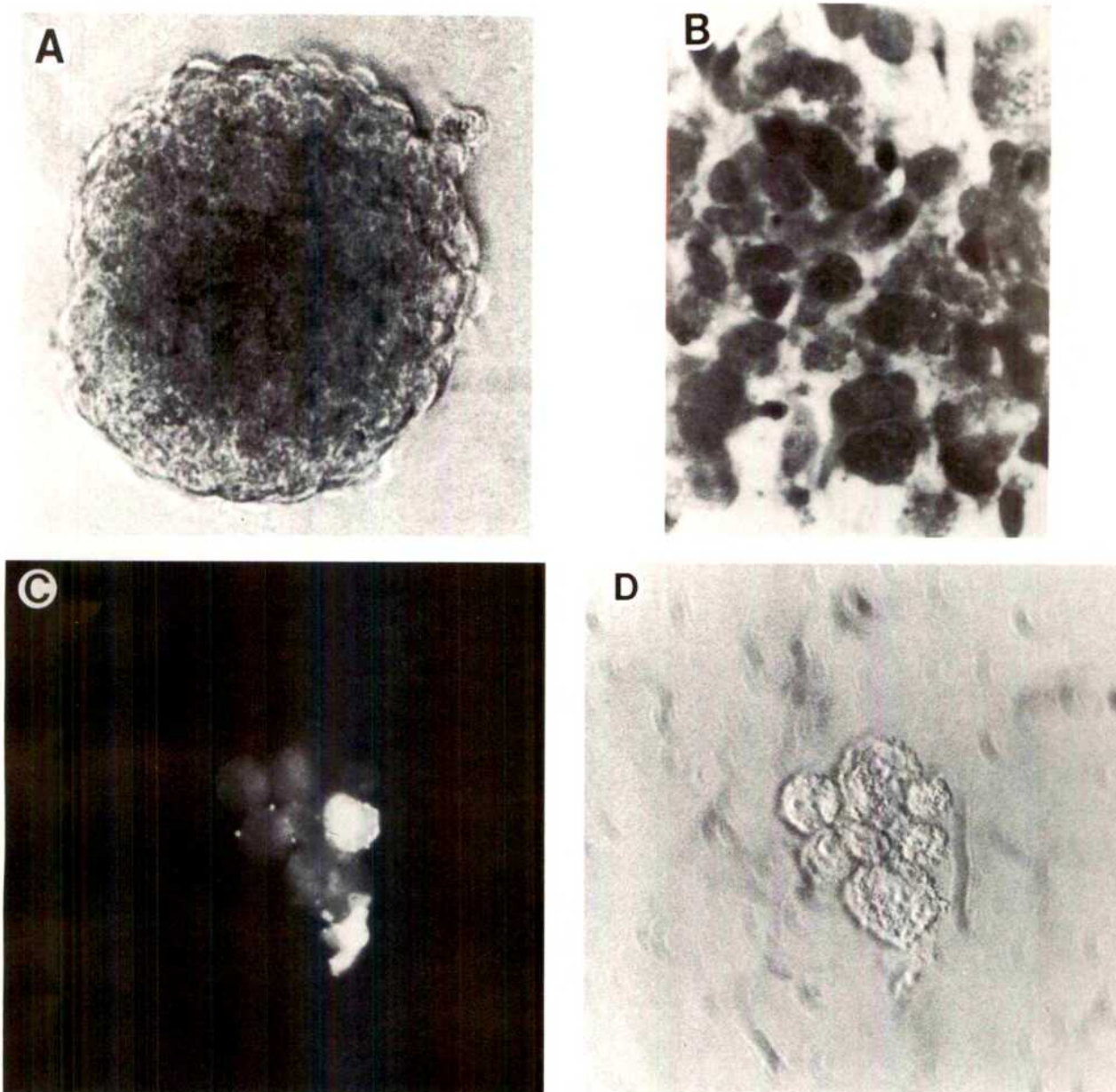


Fig 4. (A) Phase-contrast photomicrograph of a colony cultured from an SV40-transformed stromal cell line in the presence of 20% PHA-LCM. (B) Wright stain of colony. (C) Indirect immunofluorescent staining with 7.2 recognizing an HLA-DR framework determinant. (D) Corresponding phase-contrast photomicrograph.

like cells and the hematopoietic determinants T-200 and CD-3 in the round cells. Thus, clonal cell lines appeared to produce cells with both hematopoietic and stromal characteristics.

Studies of other cell lineages transformed with SV40 virus have shown that, in general, the resultant cell lines were similar to the starting cell populations.⁴²⁻⁴⁵ For example, although SV40-transformed human epidermal cells differed from nontransformed cells in growth properties and had decreased expression of keratins and cross-linked envelopes, their growth remained anchorage independent, and they were not tumorigenic in athymic nude mice.⁴² Transformed

human keratinocytes reexpressed certain characteristics of fetal cells including keratin polypeptides found in fetal epidermis.⁴⁵ SV40-transformed marrow or skin fibroblasts do not produce round cells or react with any of the antibodies to hematopoietic determinants shown in Table 2 (unpublished data). Thus, the finding of obligate, apparent multilineage stromal and round cell differentiation in SV40-transformed cell lines arising from LTMC adherent cells suggests that this differentiative program is a normal property of the cells in LTMC susceptible to SV40 transformation.

The morphological appearance of the SV40-transformed

Table 5. Colony Formation by SV40-Transformed Stromal Cell Lines

Cell Line	Colonies/5,000 Cells (20% PHA-LCM)	Plating Efficiency (%)
10A	56 ± 8.5	1.1
13	113 ± 19	2.3
9	555 ± 220	11.1
14	558 ± 160	11.2

lines described in the present report suggests that these are different from lines transformed by a recombinant plasmid of SV40.¹² Unlike the present cells, the plasmid-transformed clones contained exclusively cells that morphologically were fibroblastic. It is possible either that different cell populations were transformed by the two methods or that intact virus is needed to effect the additional differentiative programs required for round cell expression.

Although the identity of the cell(s) transformed by SV40 remains unknown, such cells were apparently present in hematopoietically inactive LTMC from two patients with aplastic anemia. The cell lines derived from these cultures contained both round cells and stromal cells and were grossly indistinguishable from similar cell lines derived from normal donors. If SV40 virus causes a cell to revert to a fetal phenotype, as suggested for keratinocytes by Bernard et al,⁴⁵ it is possible that in our system there is selective transformation of cells present in adult marrow analogous to the fetal

Table 6. Colony and Cluster Dose Response With Recombinant GM-CSF

GM-CSF (U/mL)	Clusters (>20 Cells)	Colonies (>50 Cells)
0	6 ± 3	0
10	14 ± 3	0
100	17 ± 3	0
1,000	20 ± 6	2 ± 1
10,000	57 ± 6	6 ± 1

Cells from cell line 9 at passage 16 were seeded at 5,000/mL in graded doses of GM-CSF (as semipurified COS cell supernatant). In this experiment, cells cultured with 20% PHA-LCM produced 252 ± 13 colonies/5,000 cells.

mesenchymal cell thought to differentiate into blood island cells in the embryonic yolk sac.⁴⁶ Moreover, the present data, like those reported previously, suggest that the stromal cells in human LTMC and some hematopoietic cells may originate from a common stem cell.^{4,20} In addition to implications for hematopoietic cell ontogeny, the results reported here indicate that SV40 transformation provides a useful method for immortalizing stromal cells from human LTMC so that these cells can be cloned and examined for their biosynthetic properties, their interaction with hematopoietic cells, and their origin in patients with neoplastic disorders.

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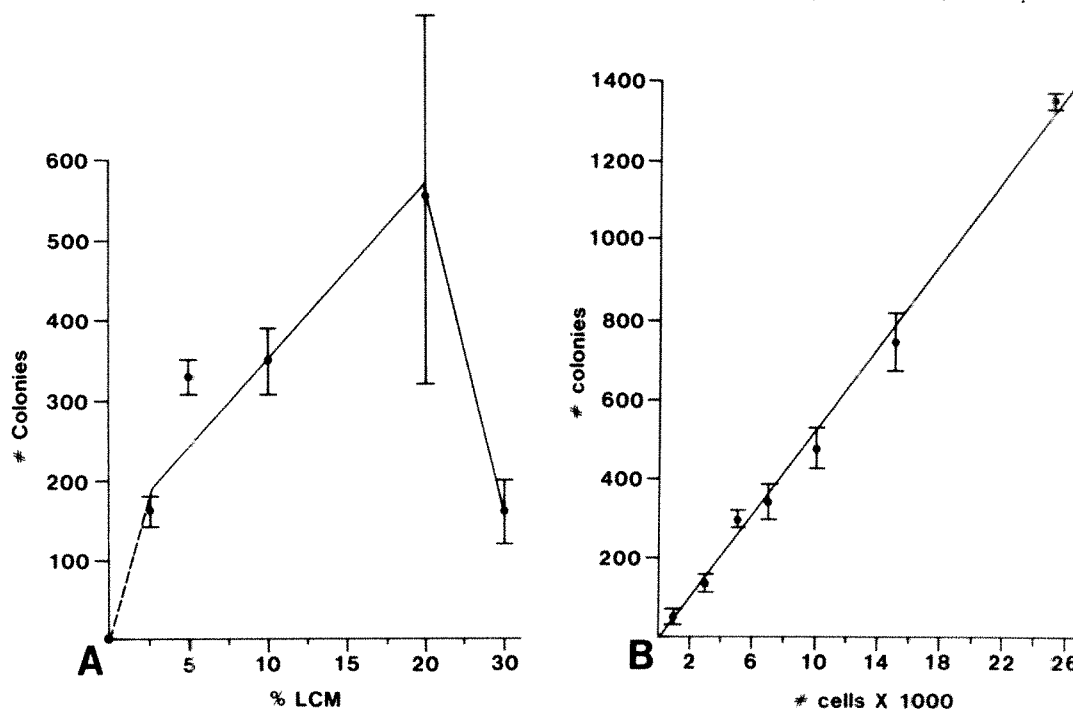


Fig 5. (A) Dose response with PHA-LCM of SV40-transformed stromal cell line 9 at the 15th passage. After trypsinization, the cells were plated at a concentration of 5,000/mL, and the number of colonies were counted under an inverted microscope at day 14. The points represent the mean ± SD of quadruplicate plates. (B) The cell dose-response relationship for cell line 9 at the 18th passage. Varying numbers of cells were plated for colony growth in the presence of 20% PHA-LCM. The seeding efficiency of cell lines decreased as they aged and in this experiment was approximately 5%.

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Increased Surface Expression of the Membrane Glycoprotein IIb/IIIa Complex Induced by Platelet Activation. Relationship to the Binding of Fibrinogen and Platelet Aggregation

By Kenji Niiya, Elisabeth Hodson, Rossella Bader, Vicky Byers-Ward, James A. Koziol, Edward F. Plow, and Zaverio M. Ruggeri

Platelet activation altered the binding of three monoclonal antibodies (monovalent Fab' fragment) directed against the glycoprotein (GP) IIb/IIIa complex. An increased binding of two- to threefold occurred after stimulation with thrombin or phorbol myristate acetate (PMA), with slight but significant increase in the dissociation constants (K_d) of two antibodies (LJ-CP8 and LJ-P9). In contrast, no statistically significant changes were observed with ADP-stimulated platelets. The increased binding of LJ-CP3, but not of the other two antibodies, to activated platelets decreased by 30% to 40% in the presence of EDTA at 22 to 25°C. Platelets stimulated by thrombin or PMA bound more fibrinogen than did those stimulated by ADP, and significant differences in the extent but not in the affinity of

fibrinogen binding were observed with various platelet agonists. When the pool of GP IIb/IIIa molecules exposed on the surface of unstimulated platelets was reacted with the monoclonal antibody LJ-CP3 to block ADP-induced fibrinogen binding and platelet aggregation, stimulation with thrombin or PMA still induced substantial binding of antibody and fibrinogen, and aggregation ensued. Therefore, platelets exposed to "strong" agonists exhibit an increased number of surface-oriented epitopes associated with GP IIb/IIIa. The GP IIb/IIIa molecules bearing these newly exposed epitopes are functional in that they can bind fibrinogen and mediate platelet aggregation.

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THE PLATELET membrane glycoprotein (GP) IIb/IIIa complex is a calcium-dependent heterodimer that serves as a receptor for molecules that mediate platelet adhesion and aggregation including fibrinogen, fibronectin and von Willebrand factor.¹⁻¹³ Occupancy of this receptor by fibrinogen is a primary pathway for platelet aggregation.¹⁴⁻¹⁷ Although GP IIb/IIIa is present on the surface of unstimulated platelets, its binding function becomes apparent only after stimulation of the cells with agonists such as ADP,¹⁴⁻¹⁷ thrombin,^{18,19} and epinephrine.^{16,20} Little is known about the mechanisms that impart this receptor activity. The two prevailing hypotheses suggest that agonists either induce a conformational transition, possibly calcium-dependent, directly within GP IIb/IIIa so as to render it a competent receptor,²¹ or cause changes in the microenvironment so that a hidden receptor becomes accessible to macromolecular ligands.^{22,23}

The transitional events that render GP IIb/IIIa a functional receptor are generally believed to be efficient. This conclusion is based on the similarity between the estimated number of fibrinogen binding sites on stimulated platelets¹⁶⁻²⁰ and that of GP IIb/IIIa molecules on the platelet surface, as determined by binding of several monoclonal antibodies to the glycoprotein.^{5-10,24} In addition to that exposed on the cell surface, other pool(s) of GP IIb/IIIa have been detected. For example, GP IIb/IIIa molecules have been identified on the intraluminal surface of vacuolar structures²⁵ which may correspond to the canalicular system of platelets.^{26,27} In intact platelets, the accessibility of this pool to antibody probes is uncertain.²⁸ In addition, by subcellular fractionation²⁹⁻³¹ and electron microscopy,²⁵ GP IIb/IIIa has been identified as a constituent of platelet α -granule membranes. When platelets are stimulated to undergo a secretory reaction, α -granule constituents, including their membrane components, as well as other intracellular species become expressed on the cell surface.^{25,32,33} Therefore, the number of GP IIb/IIIa molecules available on the surface of unstimulated and stimulated platelets may be different, but this has not been quantitatively estimated so far. Furthermore, it is unclear whether the endogenous pools of GP IIb/IIIa can serve as functional

receptors for extracellular fibrinogen or other adhesive proteins. In view of these issues, we assessed on a quantitative basis the expression of GP IIb/IIIa and functional fibrinogen receptors on the surfaces of unstimulated and stimulated platelets. The results indicate the occurrence of quantitative and qualitative changes in GP IIb/IIIa epitopes, and concomitant changes in the number of fibrinogen receptors that are dependent on the agonist used.

MATERIALS AND METHODS

Monoclonal antibody and Fab' fragment preparation. The preparation and characterization of monoclonal antibody LJ-P9 was previously reported.¹⁰ Antibodies LJ-CP3 and LJ-CP8 were prepared and characterized as described for LJ-P9, but the human washed platelets used as immunogen were treated with chymotrypsin³⁴ before injection. All three monoclonal antibodies were directed against GP IIb/IIIa and were of the "complex-specific" variety. This was shown by (a) lack of reactivity with platelets from patients with Glanzmann thrombasthenia; (b) solid-phase immunoisolation studies¹⁰ demonstrating that the antibodies reacted with platelet membrane glycoproteins having the electrophoretic mobility of GP IIb and GP IIIa; and (c) lack of reactivity with normal platelets incubated with 5 mmol/L of EDTA for 15 minutes at 37°C.³⁵⁻³⁹

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LJ-CP3 and LJ-CP8 were of the IgG₁ subclass, whereas LJ-P9 was an IgG_{2b}. Monoclonal IgG was purified from ascitic fluid using affinity chromatography on protein A Sepharose⁴⁰ (Sigma Chemical, St Louis) or by chromatography on Affi-gel blue⁴¹ (Bio-Rad, Richmond, CA). Bivalent F(ab')₂ fragments were prepared by pepsin digestion,⁴² and monovalent Fab' fragments were prepared by reduction and S-carboxymethylation of the former.¹⁰ Monoclonal IgG was also purified from an anti-GP Ib antibody, LJ-Ib 1. This antibody was characterized in a recent publication.⁴³ Protein concentration of the monoclonal antibodies was measured with the Bio-Rad assay kit using bovine IgG as a standard (Miles Scientific, Naperville, IL).

Purification of fibrinogen. Purification of fibrinogen was accomplished with the method of Kazal and colleagues⁴⁴ or Doolittle and associates.⁴⁵ All preparations were >94% clottable and had intact subunit composition when characterized as previously reported.⁴⁶

Radioiodination of proteins. Purified proteins were radiolabeled with carrier-free Na¹²⁵I or Na¹³¹I (The Radiochemical Center, Amersham, Arlington Heights, IL) using Iodogen (Pierce Chemical, Rockford, IL), as described by Fraker and Speck,⁴⁷ or by a modified chloramine-T procedure.⁴⁸ Specific activities were between 0.5 and 2 mCi/mg protein for all labeled molecules, and the radioactivity was >90% precipitable in 10% trichloroacetic acid.

Preparation of washed platelets. Platelet-rich plasma (PRP) was obtained from blood collected in acid-citrate-dextrose (ACD) anticoagulant, as described.¹¹ Platelets were washed by the albumin density-gradient technique of Walsh and colleagues,⁴⁹ with modifications previously reported,¹⁰ or by gel filtration as described by Marguerie and associates.¹⁵ Similar results were obtained with platelets prepared by both methods of purification. Washed platelets were resuspended in modified Tyrode's buffer containing 2% bovine serum albumin (BSA) (Calbiochem, La Jolla, CA), with or without added divalent cations.

Binding studies. The binding of monoclonal antibody to unstimulated or stimulated platelets was measured as reported previously.¹⁰ Platelet stimuli included: α -thrombin or γ -thrombin, the latter with a specific coagulant activity of only 0.5625 NIH U/mg (both obtained as a gift of Dr J. W. Fenton, II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY), ADP, epinephrine, and 12-O-tetradecanoylphorbol 13-acetate (phorbol myristate acetate, PMA) (all purchased from Sigma). When thrombin was used, its activity was blocked with hirudin (Sigma) following the initial incubation with platelets and before the addition of other reagents. The binding of fibrinogen to platelets was measured as reported elsewhere^{10,15} in the presence of 2 mmol/L of CaCl₂ or 2 mmol/L of MgCl₂ or 1 mmol/L of MgCl₂ and 1 mmol/L of CaCl₂. The dissociation constant (K_d) and number of binding sites were calculated by means of Scatchard-type analysis using the computer-assisted program Ligand.⁵⁰ Comparisons of K_d and number of binding sites determined under different conditions were performed with paired sample *t* tests and one- and two-way analyses of variance (ANOVA).⁵¹

In selected experiments, the ¹²⁵I-labeled fibrinogen bound to thrombin-stimulated platelets was characterized by electrophoresis on polyacrylamide gels followed by autoradiography. The platelet-bound fibrinogen was solubilized with 40 μ L of 0.02 mol/L sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate (SDS), 1 mmol/L of EDTA, 1 mmol/L of phenylmethylsulfonyl fluoride, and 15 mmol/L of dithiothreitol. After incubation at 56°C for 20 minutes, the sample was analyzed by polyacrylamide gel electrophoresis (PAGE) in the buffer system of Weber and Osborn,⁵² using 5% acrylamide and 0.14% bis acrylamide.

Preparation of monoclonal antibody-coated platelets. Platelets in Tyrode's buffer containing 2 mmol/L of MgCl₂, pH 7.4, were

incubated with LJ-CP3 F(ab')₂ at a final concentration of 1.5 to 2.5 mg/mL, for 30 minutes at 22°C. Bivalent F(ab')₂ fragments were used in these experiments because of their higher affinity for platelets than monovalent Fab' fragments, as determined in preliminary experiments. The high concentration of antibody was used to ensure saturation of all recognized epitopes in the presence of $>1.5 \times 10^{12}$ platelets/L. The absence of nonspecific effects due to free monoclonal IgG in solution was demonstrated by adding the same or higher amounts of monoclonal anti-GPIb antibodies⁴⁵ to PRP and showing that they had no effect on ADP- and γ -thrombin-induced aggregation. In some experiments, the platelets were coated with ¹³¹I-labeled antibody (added at a concentration of 1.1 mg/mL) to monitor the amount of bound antibody throughout the procedure. Antibody-coated platelets were then separated from unbound antibody by passage through a column of Sepharose 2B CL (Pharmacia Fine Chemicals, Piscataway, NJ) in Tyrode's buffer, pH 7.4, containing 2 mmol/L of MgCl₂ and 2% BSA. Immediately after collection, <1% of the antibody present in the platelet fractions was unbound, as judged by the capacity of cosedimenting tracing amounts of radiolabeled F(ab')₂ from the platelet suspension using centrifugation. The antibody-coated platelets were tested for their binding of fibrinogen or LJ-CP3 F(ab')₂ before and after stimulation, or diluted into citrated (0.011 mol/L) platelet-poor plasma (PPP) from the original donor for aggregation studies. Platelet aggregation was tested as described¹⁰ using ADP, ADP plus epinephrine, PMA and γ -thrombin, rather than α -thrombin, to avoid the clotting of plasma.

RESULTS

Monoclonal antibody binding to unstimulated and stimulated platelets. Three monoclonal antibodies were used in this study to characterize GP IIb/IIIa on unstimulated and stimulated platelets. Each of these antibodies bound to platelets only when the integrity of the GP IIb/IIIa complex was maintained. This was shown for LJ-P9 in a previous publication,¹⁰ and similar properties were also observed for LJ-CP3 and LJ-CP8. Namely, the binding of these radiolabeled antibodies to platelets, whether stimulated or not, was decreased by >80% when the platelets were preincubated with 5 mmol/L of EDTA for 30 minutes at 37°C, pH 7.4, as compared with their binding in the presence of Ca²⁺.

Binding of these three antibodies to unstimulated and thrombin-stimulated platelets was examined under various divalent ion conditions at 22 to 25°C. As shown in Fig 1, thrombin stimulation of platelets caused an increase in the binding of LJ-CP3 and LJ-CP8. This increase in binding was observed in the presence of Ca²⁺, Mg²⁺, and EDTA. At the concentrations used, this increase was more marked with LJ-CP3 than with LJ-CP8. In addition, the binding of LJ-CP3 to thrombin-stimulated platelets was less with EDTA than with Ca²⁺ and Mg²⁺. This was in contrast to the observation that the binding of LJ-CP8 to thrombin-stimulated platelets was of a similar extent in the presence of Ca²⁺ and EDTA (Fig 1). LJ-P9 behaved like LJ-CP8 in this regard (not shown).

To investigate further the effect of thrombin stimulation on the interaction of the monoclonal antibodies with GP IIb/IIIa, the binding of their Fab' fragments to thrombin-stimulated and unstimulated platelets was compared as a function of antibody concentration. All three antibodies showed clear evidence of saturable binding to the thrombin-

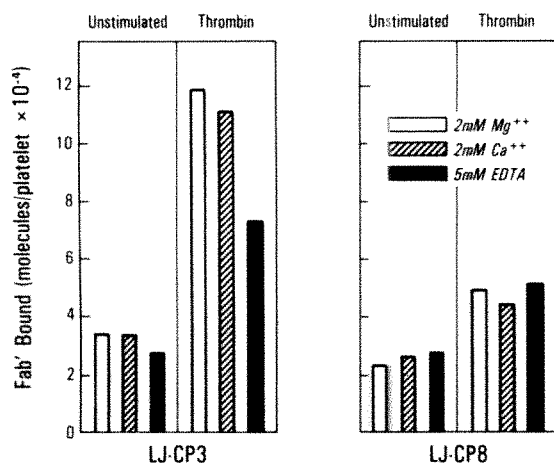


Fig 1. Effect of EDTA and divalent cations on anti-GP IIb/IIIa Fab' fragment binding. Washed platelets at a count of 2.5×10^8 /mL were stimulated with 0.1 NIH U/mL of α -thrombin for 10 minutes at 22 to 25°C. Modified Tyrode's buffer was used instead of thrombin in the control (unstimulated) mixture. Hirudin (or the same volume of buffer) was then added in a 12-fold excess (U/U) and incubated for 5 minutes, followed by ^{125} I-labeled Fab' fragment for an additional 30 minutes. Platelet count at this point was 1×10^8 /mL. The experimental mixture contained either 2 mmol/L of $MgCl_2$ or 2 mmol/L of $CaCl_2$, or 5 mmol/L of Na_2 EDTA, as shown. The binding of LJ-CP3 or LJ-CP8 was measured following sedimentation of the platelets by centrifugation through a 20% sucrose layer. The concentrations of added antibodies (30 μ g/mL for LJ-CP3 and 20 μ g/mL for LJ-CP8) approached saturating amounts. Incubations were performed at 22 to 25°C. LJ-CP3 bound to more epitopes than did LJ-CP8, particularly after platelet stimulation with thrombin, as clearly shown also in Fig 2 and Table 1.

stimulated and unstimulated platelets (Fig 2). With all three antibodies, the maximal binding observed with thrombin-stimulated platelets was significantly greater ($P < .001$) than that observed with the unstimulated cells. This was most evident with LJ-CP3, followed in decreasing order by LJ-P9 and LJ-CP8. The K_d of LJ-CP3 tended to be lower after thrombin stimulation, although the change was not statistically significant; in contrast, the K_d of LJ-CP8 and LJ-P9 were significantly higher ($P < .01$) with thrombin-stimulated platelets than with unstimulated platelets, thereby possibly explaining why single concentrations of the antibodies tested at or below their K_d for unstimulated platelets readily demonstrated increased binding to thrombin-stimulated platelets with LJ-CP3, but less clearly with the other two antibodies (Figs 1 and 2). Figure 2 shows that at the highest concentration of each antibody added, the increase in Fab' molecules bound to thrombin-stimulated relative to unstimulated platelets was 263% for LJ-CP3, 212% for LJ-CP9, and 200% for LJ-CP8.

The results of several experiments performed with platelets from different donors are summarized in Table 1. A certain degree of variability was found in the extent of antibody binding to different individual platelets, with a broader range (molecules per platelet) for LJ-CP3 (resting: 33,500 to 81,100; thrombin-stimulated: 88,600 to 196,000) and LJ-CP8 (resting: 27,500 to 67,100; thrombin-stimulated: 45,200 to 131,900) than for LJ-P9 (resting: 42,400 to

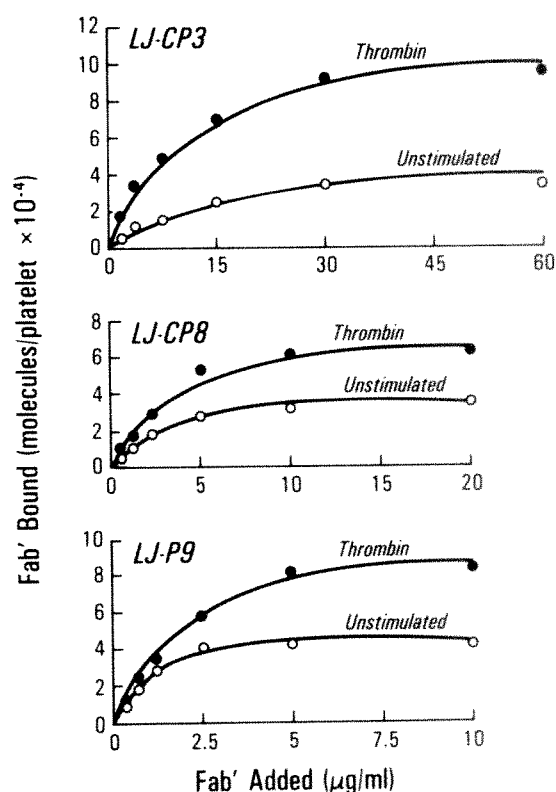


Fig 2. Binding of anti-GP IIb/IIIa monoclonal Fab' fragments to unstimulated and stimulated platelets. Washed platelets were stimulated with thrombin as described in the legend to Fig 1. Increasing amounts of antibody (monovalent Fab' fragment) were then added, in the presence of 2 mmol/L of Ca^{2+} and 2 mmol/L of Mg^{2+} . Binding was calculated on the basis of the specific activity by means of the computer-assisted program Ligand.⁵⁰

55,200; thrombin-stimulated: 102,400 to 113,900). The increased antibody binding was specific for GP IIb/IIIa, since binding of a monoclonal antibody to another major membrane glycoprotein, GP Ib, was not increased after thrombin stimulation (Table 1). This observation excludes differences in the recoveries of stimulated and unstimulated platelets in the binding assays. In contrast to the results observed with thrombin, stimulation of platelets with ADP, at concentrations that caused maximal fibrinogen binding with this agonist, yielded no statistically significant changes in the binding of LJ-CP3 as compared with unstimulated platelets (Table 1). Increased binding of antibody, as well as binding of fibrinogen, occurred only as a consequence of direct stimulation of platelets. Addition of the supernatant of thrombin-stimulated platelets to resting platelets, after neutralization of thrombin with hirudin, did not cause either increased binding of LJ-CP3 or binding of fibrinogen. This observation excludes the possibility that products released from platelets altered the antibodies or fibrinogen and thereby increased their binding.

Fibrinogen binding to platelets stimulated with different agonists. As expected, the amount of ^{125} I-fibrinogen bound to stimulated platelets was significantly greater than that to unstimulated platelets ($P < .001$) regardless of agonist used. Significant differences, however, occurred in the amount of

Table 1. Binding of Three Anti-GP IIb/IIIa Monoclonal Antibodies to Unstimulated and Thrombin-Stimulated Platelets

Antibody	n	Stimulus	Bound (Molecules/Platelets)	K _d (mol/L)
Anti-GP IIb/IIIa				
LJ-CP3	7	—	56,912 ± 18,205	2.56 ± 1.1 × 10 ⁻⁷
	7	Thrombin	131,288 ± 36,874*	1.98 ± 0.42 × 10 ⁻⁷
	3	ADP	68,562 ± 14,000	1.67 ± 0.29 × 10 ⁻⁷
LJ-CP8	8	—	47,495 ± 14,306	0.94 ± 0.25 × 10 ⁻⁷
	8	Thrombin	89,169 ± 25,250*	1.38 ± 0.27 × 10 ⁻⁷ †
LJ-P9	4	—	49,730 ± 5,443	3.00 ± 0.84 × 10 ⁻⁸
	4	Thrombin	110,339 ± 5,355*	4.83 ± 1.29 × 10 ⁻⁸ †
Anti-GPIIb				
LJ-Ib1	3	—	40,317 ± 11,853	4.86 ± 1.87 × 10 ⁻⁷
	3	Thrombin	30,817 ± 18,029	3.56 ± 2.57 × 10 ⁻⁷

Parameters of binding were calculated by Scatchard-type analysis of binding isotherms using the computer-assisted program Ligand⁶⁰ and are reported as mean ± SD. α -Thrombin concentration was 0.1 NIH U/mL, and ADP was 10 μ mol/L.

Asterisk and dagger indicate values obtained with stimulated platelets that were significantly greater than corresponding values obtained with unstimulated platelets (paired comparison *t* test).

**P* < .001.

†*P* < .01.

bound fibrinogen with different agonists; α -thrombin and PMA induced greater binding than either ADP or epinephrine (*P* < .05; Table 2). In further experiments, saturation curves of fibrinogen binding to ADP- and PMA-stimulated platelets were compared with four different donors and evaluated by Scatchard-type analysis. In these studies, PMA was used as an agonist, rather than thrombin, to avoid fibrin formation due to any residual thrombin activity. At saturation, the binding of fibrinogen (mean ± SD) to platelets stimulated with PMA (10 nmol/L) was 76,100 ± 13,200 molecules/platelet (range: 58,500 to 87,900) and was significantly greater (*P* < .001) than its binding to platelets stimulated with ADP (10 μ mol/L), 17,600 ± 9,100 molecules/platelet (range: 9,800 to 30,600). On the other hand, dissociation constants, K_d = 4.78 ± 3.9 × 10⁻⁷ mol/L for PMA and K_d = 5.78 ± 2.6 × 10⁻⁷ mol/L for ADP, were not significantly different. Furthermore, the increase in fibrinogen binding to thrombin-stimulated platelets (Table 2) did not appear to arise as a result of fibrin formation. Qualitative analysis of the fibrinogen bound to thrombin-stimulated platelets was performed by SDS-PAGE after reduction of disulfide bonds (Fig 3). Under conditions in which the α and β chains of fibrin could be distinguished from the A α and B β

chains of fibrinogen, the platelet bound ligand contained intact A α and B β chains.

Aggregation and binding studies with antibody-coated platelets. To evaluate whether the increased surface expression of GP IIb/IIIa molecules following platelet stimulation with certain agonists was paralleled by an increase in

Table 2. Binding of ¹²⁵I-Fibrinogen to Platelets Stimulated With Different Agonists

Agonist	Fibrinogen Bound (Molecules/Platelet)
ADP (10 μ mol/L)	38,600 ± 17,300
α -thrombin (0.1 NIH U/mL)	90,800 ± 37,900
PMA (10 nmol/L)	80,000 ± 47,200
Epinephrine (10 μ mol/L)	30,200 ± 11,900

Experiments were performed in three different normal donors using a saturating amount of ¹²⁵I-fibrinogen (1 mg/mL final concentration) and the indicated final concentrations of different agonists. Results represent the mean ± SD. Values for ADP and epinephrine were significantly lower than values for α -thrombin and PMA (two-way analysis of variance: *P* < .05).

Abbreviation: PMA, 12-O-tetradecanoylphorbol 13-acetate (phorbol myristate acetate).

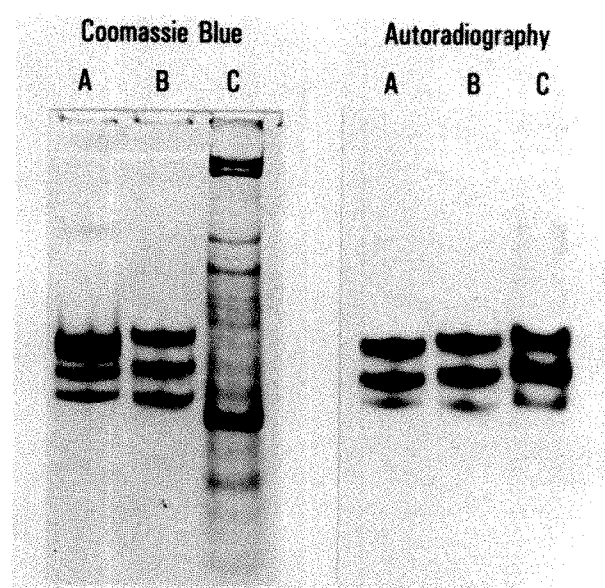


Fig 3. Electrophoretic analysis of ¹²⁵I-labeled fibrinogen bound to thrombin-stimulated platelets. The three lanes on the left show Coomassie blue staining of: fibrin (A), generated by incubating purified fibrinogen (1 mg/mL) with 1 NIH U/mL of α -thrombin in the presence of 2 mmol/L of EDTA; purified fibrinogen (B); lysed platelets after separation from unbound fibrinogen (C), as described in the Materials and Methods section. The fibrinogen used contained 1% of the total amount as ¹²⁵I-labeled fibrinogen. The three lanes on the right show an autoradiography of the same gel shown on the left. Cathode is shown at top. The migration rate of the fibrin α and β chains was increased as compared with the corresponding fibrinogen A α and B β chains. The mobility of platelet-bound fibrinogen corresponds to that of purified fibrinogen. Photographs are from an uncut gel.

functional fibrinogen binding sites, we performed experiments in which unstimulated platelets were coated with LJ-CP3 F(ab')₂ and then tested in aggregation studies after removal of any unbound antibody. In this manner, the function of the pool of GP IIb/IIIa exposed on the surface of unstimulated platelets was largely blocked and that of the newly expressed GP IIb/IIIa molecules became more apparent. Control platelets were always prepared at the same time as antibody-coated platelets. These control platelets were treated identically as the experimental set except that the antibody was omitted, and they served as a means of assuring the function of the platelets throughout the procedure. In interpreting the results of these experiments, one should consider that the binding of antibody to platelets was reversible (Fig 4). This finding explains why platelets treated with saturating amounts of antibody and then gel-filtered could still bind newly added antibody. The values shown in Table 3 (comparing LJ-CP3 binding to unstimulated, control, and treated platelets) agree with the dissociation of ~30% to 50% of previously bound antibody, which typically occurred in 30 to 60 minutes (Fig 4).

Aggregation in response to ADP was almost completely abolished with antibody-coated platelets, whereas that in response to ADP plus epinephrine, γ -thrombin and PMA was still present, although variably reduced as compared with control platelets (Fig 5). The least reduction occurred when γ -thrombin was used as agonist. The aggregation of antibody-coated platelets, regardless of the agonist used, was completely inhibited by the addition to the suspension of saturating amounts of the monoclonal antibody LJ-CP3, thus demonstrating that GP IIb/IIIa was the receptor involved in the residual aggregation observed. The same

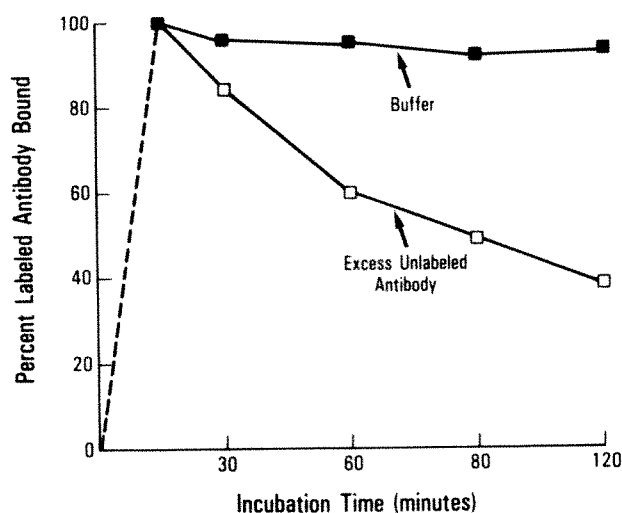


Fig 4. Dissociation of bound LJ-CP3 F(ab')₂. Platelets washed by the albumin density-gradient technique (final count 1×10^8 /mL) were mixed with ¹²⁵I-labeled bivalent F(ab')₂ of monoclonal antibody LJ-CP3 (final concentration 10 μ g/mL). After incubation for 15 minutes at 22 to 25°C, a 100-fold excess of unlabeled F(ab')₂ was added (or the same volume of Tris buffer in a control mixture). Residual binding was measured at several time points and expressed as a percentage of the binding measured before addition of unlabeled ligand.

antibody-coated platelets, resuspended in Tyrode's buffer rather than plasma, were used to measure the binding of newly added LJ-CP3 F(ab')₂ and fibrinogen after stimulation. The binding of antibody to the stimulated coated platelets revealed an increase that, in terms of molecules bound per platelet, was similar to that which occurred with the control platelets, depending on the agonist used. Thus, the increase was minimal with ADP and maximal with thrombin and PMA (Table 3). The binding of fibrinogen to coated platelets stimulated with ADP or ADP plus epinephrine was extremely reduced, whereas coated platelets stimulated with thrombin or PMA bound a number of fibrinogen molecules similar to the number of newly exposed GP IIb/IIIa molecules detected with the monoclonal antibody (Table 3).

To demonstrate that free antibody remaining in or released into the suspending medium did not affect the results of aggregation and fibrinogen binding studies, at the end of each experiment the antibody-coated platelets were removed by centrifugation and the supernatant was tested for any inhibitory effect on platelet aggregation. No such effect could be demonstrated. Moreover, it is possible to calculate that a dissociation corresponding to 50% of previously bound antibody would result in a free antibody concentration of <0.5 μ g/mL (assuming 25,000 molecules of bivalent F(ab')₂ released per platelet, a count of 1×10^8 platelet/mL and a mol wt for the antibody fragment of 110 kd). This concentration of LJ-CP3 has no detectable effect on platelet function or fibrinogen binding.

As a further control for these experiments with antibody-coated platelets, the GP IIb/IIIa molecules exposed on the surface of unstimulated platelets were saturated with ¹³¹I-labeled LJ-CP3 F(ab')₂ and then reacted, before or after stimulation, with ¹²⁵I-labeled antibody. The experiment was performed with a limited number of samples to minimize the time of execution. As a consequence, the dissociation of bound antibody was also reduced, thus explaining the lesser binding of ¹²⁵I-labeled antibody to coated platelets observed in this experiment as compared with those reported in Table 3. The results shown in Fig 6 demonstrate that thrombin-stimulated platelets, unlike resting platelets, bound a significant amount of ¹²⁵I-labeled antibody at a time when the pool of GP IIb/IIIa molecules exposed on unstimulated platelets was still blocked by the ¹³¹I-labeled antibody. The minimal binding of ¹²⁵I-labeled antibody to the resting coated platelets can be explained by occupation of sites from which ¹³¹I-labeled antibody had dissociated. The modest increase in binding of the ¹²⁵I-labeled antibody to ADP-stimulated platelets is consistent with the increase observed in all experiments with antibody LJ-CP3 (Table 1).

DISCUSSION

The present studies define in a quantitative manner the increase in the expression of GP IIb/IIIa-related epitopes on the platelet surface following activation with strong agonists. The number of GP IIb/IIIa molecules exposed on the membrane of unstimulated platelets has been previously determined by measuring the binding of monoclonal antibody-

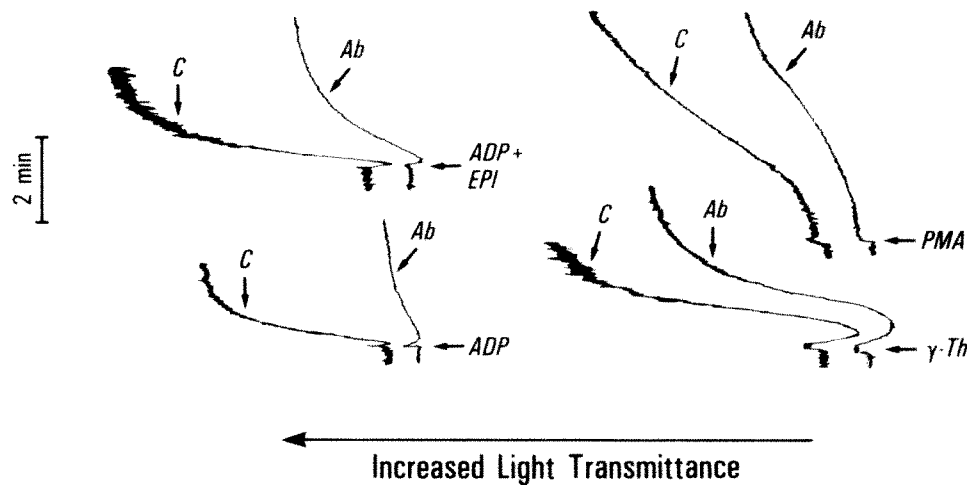


Fig 5. Aggregation of antibody-coated platelets. Each pair of curves shows the aggregation of platelets coated with LJ-CP3 F(ab')₂ fragment (Ab, preparation described in the Materials and Methods section) as compared with control platelets (C), both resuspended in citrated platelet-poor plasma from the same donor (platelet count 2×10^9 /mL). Four different agonists were used, as shown, at the following concentrations: ADP, 5 μ mol/L; ADP + epinephrine (EPI), 5 μ mol/L each; γ -thrombin (Th), 50 μ g/mL; phorbol myristate acetate (PMA), 10^{-8} mol/L. The reduction of aggregation (based on changes in light transmittance) of antibody-coated platelets as compared with control platelets was: 90% with ADP; 70% with ADP + Epi; 40% with PMA; and 33% with γ -thrombin. Control platelets were treated like coated platelets except that they were incubated with buffer, rather than antibody, before the gel filtration (described in the Materials and Methods section).

ies,^{5,10,21-24} and the results presented here are well in line with those published by other researchers. We found variability in the binding of the same antibody to different platelets, a result already reported in previous studies^{8,10} and one that likely reflects individual variations in the content of platelet GP IIb/IIIa. We also found differences in the binding of distinct antibodies to the same unstimulated platelets, a result that may reflect variations in the steric constraints imposed on distinct epitopes of some GP IIb/IIIa molecules.

Until recently, less attention was devoted to measuring the binding of monoclonal anti-GP IIb/IIIa antibodies to stimulated platelets. Some studies^{6,22,23} were performed with ADP-stimulated platelets and, as in the present report, no significant differences in antibody binding were found with this agonist as compared with unstimulated platelets. The binding of one monoclonal antibody (B 59.2) was identical for platelets either unstimulated or stimulated with ADP, collagen, or arachidonic acid.⁵ PAC-I,²¹ an IgM antibody that

interacts only with stimulated platelets, bound more to thrombin- than to ADP- or epinephrine-stimulated platelets (20,000 to 25,000 v 10,000 to 15,000 molecules/platelet, respectively), an observation consistent with the results of the present study. Indeed, the present study clearly demonstrates a two- to threefold increase in the binding of monoclonal anti-GP IIb/IIIa antibodies (of the IgG class) to platelets activated with strong stimuli as compared with resting platelets. This does not appear to be a nonspecific consequence of morphological or metabolic changes occurring with platelet activation, since another major membrane GP, GP Ib, did not show any changes similar to those observed for GP IIb/IIIa. George and colleagues also recently reported⁵³ an increase in GP IIb/IIIa epitopes on thrombin-stimulated platelets but did not address the functional properties of the newly expressed GP IIb/IIIa molecules.

From the present studies, analysis of binding isotherms, rather than measurements performed at single ligand con-

Table 3. Binding of Monoclonal Antibody LJ-CP3 and Fibrinogen to Antibody-Coated and Control Platelets

Agonist	¹²⁵ I-LJ-CP3 Bound (Molecules/Platelet $\times 10^{-3}$)		¹²⁵ I-Fibrinogen Bound (Molecules/Platelet $\times 10^{-3}$)	
	Control	Treated	Control	Treated
ADP (10 μ mol/L)	58.7 $\pm$ 9.5 (9.0)	25.3 $\pm$ 9.4 (3.1)	17.2 $\pm$ 4.2 (13.3)	4.3 $\pm$ 0.77 (0)
ADP + epinephrine (10 μ mol/L each)	65.2 $\pm$ 13.1 (15.5)	36.7 $\pm$ 9.4 (14.5)	48.3 $\pm$ 10.8 (44.4)	7.6 $\pm$ 0.63 (3.3)
α -Thrombin (0.1 NIH U/mL)	110.6 $\pm$ 30.0 (60.9)	65.9 $\pm$ 12.9 (43.7)	118.4 $\pm$ 2.3 (114.5)	39.2 $\pm$ 18.1 (34.9)
PMA (10 nmol/L)	101.7 $\pm$ 26.6 (52.0)	64.7 $\pm$ 20.1 (42.5)	118.9 $\pm$ 23.7 (115.0)	46.1 $\pm$ 6.4 (41.8)
Unstimulated	49.7 $\pm$ 11.1	22.2 $\pm$ 12.6	3.9 $\pm$ 0.47	4.3 $\pm$ 1.5

Preparation of antibody-coated platelets is described in the text. Results shown are the mean $\pm$ SD of four experiments with LJ-CP3 and three with fibrinogen. Values in parentheses represent the difference in molecules bound ($\times 10^{-3}$) between stimulated platelets and the corresponding unstimulated platelets. The final concentrations used in the binding assay were 15 μ g/mL of bivalent F(ab')₂ and 1 mg/mL of fibrinogen.

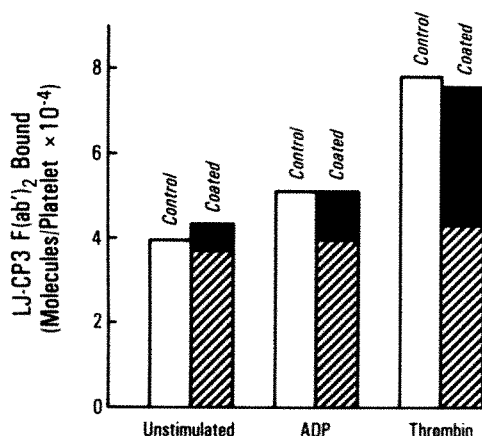


Fig 6. Binding of ^{125}I -labeled antibody to resting or stimulated platelets coated with ^{125}I -labeled antibody. Open columns indicate the binding of ^{125}I -labeled LJ-CP3 F(ab')_2 (added at a concentration of $50 \mu\text{g/mL}$) to control platelets, either unstimulated or stimulated with ADP ($10 \mu\text{mol/L}$) or α -thrombin (0.1 NIH U/mL), as indicated. Shaded columns indicate the amount of antibody remaining bound to platelets coated with ^{125}I -labeled LJ-CP3 F(ab')_2 , prepared as described in the Materials and Methods section. Solid columns indicate the additional amount of ^{125}I -labeled antibody bound to the coated platelets, either resting or stimulated. The increased binding to thrombin-stimulated platelets corresponds to a pool of GP IIb/IIIa molecules that were not accessible for binding the antibody when in the resting state.

centrations, clearly is essential to detect the increased binding of anti-GP IIb/IIIa antibodies. This may be due to changes in the affinity of the interaction between antibody and activated platelets. Indeed, two of the three antibodies tested (LJ-CP8 and LJ-P9) had significantly lower affinity for activated platelets. When tested at single concentrations, corresponding approximately to the K_d of the interaction, the binding of these two antibodies to activated platelets was less clearly increased than that of LJ-CP3, whose affinity for activated platelets was not decreased. Moreover, divalent cation conditions are important in modulating the binding of some monoclonal antibodies to activated platelets, as shown by the fact that the binding of LJ-CP3 to thrombin-stimulated platelets was partially inhibited by EDTA, even at 22 to 25°C . Adequate consideration must be given to all these points for detecting the increased binding of monoclonal anti-GP IIb/IIIa antibodies to activated platelets. Moreover, certain epitopes appear to be more accessible than others to antibody probes, since LJ-CP3 binding to both resting and stimulated platelets was greater than that of the other two antibodies.

The greater number of GP IIb/IIIa epitopes exposed on the membrane of thrombin-stimulated or PMA-stimulated platelets may be the consequence of translocation of molecules from intracellular pools,^{29,32} an event demonstrated by immunofluorescence and immunoelectron microscopy for GP IIb/IIIa in the α -granule membrane,²⁵ or may represent the "opening" of surface-connected compartments that may be inaccessible to immunologic probes in resting platelets,^{25,28,32} or may be a combination of the two. It is also possible, although less likely, that the increase in GP IIb/IIIa-related epitopes following activation of platelets occurs

without changes in the actual number of molecules, as a consequence of conformational changes in or around GP IIb/IIIa.

In the present series of experiments, fibrinogen binding to platelets exposed to strong stimuli such as thrombin or PMA was greater than previously reported. It is unlikely that this is the consequence of changes in the ligand rather than in the number of receptor molecules, at least as judged by the lack of changes in the electrophoretic mobility of the $\text{A}\alpha$ and $\text{B}\beta$ chains of bound fibrinogen, suggesting that no fibrin generation had occurred either before or as a consequence of binding. Moreover, we demonstrate here that stimuli such as thrombin lead to the surface expression of GP IIb/IIIa molecules in addition to those present on the membrane of resting platelets. Accordingly, the number of fibrinogen molecules bound after exposure to thrombin or PMA is close to the total number of GP IIb/IIIa molecules present on the surface of these activated platelets, as determined by antibody binding. These newly exposed molecules can function in binding fibrinogen and mediating platelet aggregation even when the pool of GP IIb/IIIa on the membrane of unstimulated platelets is functionally inhibited by a specific monoclonal antibody, as shown in the experiments performed with antibody-coated platelets stimulated in the absence of free antibody. Therefore, the greater binding of fibrinogen to platelets exposed to strong agonists is reasonably explained by the increased availability of receptor molecules.

On the other hand, ADP-induced fibrinogen binding and platelet aggregation are almost entirely dependent on the receptor function acquired by the GP IIb/IIIa molecules on the surface of unstimulated platelets, in agreement with the observation that this agonist has only a limited effect in increasing the number of GP IIb/IIIa molecules expressed on the platelet membrane. Variations in the number of fibrinogen molecules bound to platelets stimulated with "weak" agonists such as ADP, as occurred in the present study as well as in previous studies,^{15-20,54} may reflect changes in the reactivity of individual preparations of washed platelets. Nevertheless, our results suggest that the difference in binding between platelets stimulated with weak and strong stimuli is the consequence of a difference in the number of available binding sites.

In conclusion, this report provides evidence for the existence of at least two compartments of GP IIb/IIIa, one of which is exposed on the surface of resting platelets and exhibits receptor function for adhesive molecules on stimulation with appropriate agonists; the other, not expressed on the membrane of unstimulated platelets, becomes exposed on activation with strong stimuli. Thus, the GP IIb/IIIa molecules in both compartments exhibit the ability to bind fibrinogen. The functional activity of the newly expressed GP IIb/IIIa molecules is relevant for enhanced fibrinogen binding capacity and aggregation of platelets in response to certain physiologic agonists such as thrombin.

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Photoradiation Models for the Clinical Ex Vivo Treatment of Autologous Bone Marrow Grafts

By Jens Atzpodien, Subhash C. Gulati, Annabel Strife, and Bayard D. Clarkson

To assess the potential of photoradiation therapy for the in vitro purging of residual tumor cells from autologous bone marrow (BM) transplants, we studied normal marrow and tumor cell clonogenicity in response to different light-activated compounds by using the fluorescent dyes dihematoporphyrin ether (DHE) and merocyanine-540 (MC-540). After photoradiation of cells with white light, both DHE and MC-540 showed high cytotoxic activity toward lymphoid and myeloid neoplastic cells but had a significantly lesser effect on normal granulocyte-macrophage (CFU-GM), erythroid (BFU-E), and mixed colony-forming (CFU-GEMM) progenitor cells. Acute promyelocytic leukemia (HL-60), non-B, non-T, CALLA-positive acute lymphoblastic leukemia (Reh), and diffuse histiocytic B cell lymphoma (SK-DHL-2) cell lines were exposed to different drug concentrations in combination with white light at a constant illumination rate of 50,000 lux. With DHE doses varying from 2.0 to 2.5 $\mu\text{g/mL}$ and MC-540 concentrations of 15 to 20 $\mu\text{g/mL}$, clonogenic tumor cells could be reduced by more than 4 logs when treated alone or in mixtures with

normal irradiated human marrow cells. However, preferential cytotoxicity towards neoplastic cells was highly dependent on the mode of light activation. MC-540 had no substantial effect on malignant lymphoid (SK-DHL-2) and myeloid (HL-60) cells and on normal marrow myeloid (CFU-GM) precursors when drug incubation was performed in the dark and followed by light exposure of washed cells. Equal doses of MC-540 (15 to 20 $\mu\text{g/mL}$) could preferentially eliminate tumor cells under conditions of simultaneous light and drug treatment (30 minutes at 37°C). When using DHE (2.5 $\mu\text{g/mL}$), 29.3%, 46.8%, and 27.5% of normal marrow CFU-GM, BFU-E, and CFU-GEMM, respectively, were spared after sequential drug and light exposure of cells, whereas simultaneous treatment reduced both normal (CFU-GM) and neoplastic cells below the limits of detection. In summary, our results indicate the usefulness of various photoradiation models for the ex vivo treatment of leukemic and lymphomatous bone marrow autografts.

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PHOTORADIATION THERAPY (PRT), combining the application of light and light-activated tumor-photosensitizing agents, is a promising new approach to cancer treatment.¹⁻³ The broad clinical use of PRT in different types of neoplasms⁴⁻⁸ has also made it a possible candidate for the ex vivo treatment of autologous bone marrow (BM) transplants. Selective methods for purging neoplastic cells from harvested marrow are being developed to increase the efficacy of autologous bone marrow transplantation (ABMT) as a rescue from myeloablative chemoradiotherapy.⁹⁻¹² In vitro purification of tumor cell-contaminated BM grafts may be achieved by preferentially removing neoplastic cells without affecting the transplant's potential for engraftment.

Experimental BM-purging models need to be evaluated with respect to the following questions:¹¹⁻¹⁵ (a) To what extent are tumor cells being eliminated in the in vitro

system? (b) Is antineoplastic activity being attained under conditions that preserve sufficient numbers of normal hematopoietic stem cells for reconstitution? (c) Does the presence of bone marrow cells interfere with cytotoxic effects on tumor cells?

Photoradiation treatment uses various sensitizing compounds, among which hematoporphyrins and merocyanines have been extensively studied.^{3-8,16-18} Dihematoporphyrin ether (DHE) and merocyanine-540 (MC-540) are fluorescent agents that preferentially localize in tumor cells and undergo photoexcitation when exposed to appropriate non-ionizing radiation.¹⁸⁻²³ These properties have been exploited to accomplish a selective tumor cell destruction in vitro.^{1-8,17,18,24} Using either DHE or MC-540 in combination with white light, we studied the dose-dependent effects of photoradiation treatment on leukemia and lymphoma cell lines as compared with various normal BM progenitor cells. Based on the results presented here, different experimental photoradiation models could be developed for future application to clinical ABMT trials.

MATERIALS AND METHODS

Cells. Normal BM was obtained by aspiration from the posterior iliac crests of healthy volunteers after informed written consent. Heparinized specimens were washed in calcium- and magnesium-free phosphate-buffered saline. BM mononuclear cells were enriched by neutral density centrifugation on a Ficoll-Hypaque gradient (density, 1.077 g/L). For photoradiation experiments, low-density cells were collected and resuspended in RPMI 1640 supplemented with 2% fetal calf serum (FCS, HyClone, Logan, UT).

For BM-purging studies, three different neoplastic cell lines were chosen: HL-60, an acute promyelocytic leukemia cell line²⁵; Reh, a CALLA-positive, non-T, non-B acute lymphoblastic leukemia cell line²⁶; and SK-DHL-2, an (8;14)-translocated diffuse histiocytic lymphoma B cell line.²⁷ All cell lines were maintained under exponential growth conditions in RPMI 1640 media supplemented with 10% FCS, 1% penicillin-streptomycin-neomycin (PSN; GIBCO, Grand Island, NY) and 1% L-glutamine and were kept at

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37°C in a humidified atmosphere of 5% CO₂ in air. Cell viability ranged from 95% to 99%. All cell lines were free of mycoplasma contamination. Before photoradiation, cells were washed and resuspended in RPMI 1640 supplemented with 2% FCS.

Reagents. DHE (Photofrin II; Photofrin Medical, Raritan, NJ) an active compound derived from hematoporphyrin derivative,¹⁹ and MC-540 (Eastman Kodak, Rochester, NY) were used as cell-photosensitizing agents. DHE was obtained as a sterile aqueous solution at 2.5 mg/mL. Stock solutions of MC-540 were prepared in 50% ethanol-water at 1 mg/mL. For each experiment, stock solutions were diluted in serum-free RPMI.

Photoradiation. Cells were exposed to white light (75-W General Electric filament light bulb, luminous flux of approximately 1,170 lumens) at a radial distance of 15 cm. Clear polystyrene test tubes (15-mm diameter) containing 2 mL of cell suspension were placed in a circular array around the light source. In all experiments, cell illumination was constant at 50,000 lux, as measured through polystyrene tubes with a calibrated Luna-Pro sbe photometer (Gossen, West Germany). The temperature of light-exposed cell suspensions was taken inside all the test tubes and was found to be 37.0 ± 0.2°C (mean ± range).

Bioassays. Exponentially growing HL-60, Reh, and SK-DHL-2 cells and normal human BM mononuclear cells were incubated with different concentrations of DHE or MC-540 at 37°C for 30 minutes. Based on preliminary experiments, two alternative modes of light exposure were chosen: (a) sequential exposure, ie, after pretreatment with DHE or MC-540 in the dark, cells were washed and subsequently exposed to white light at 50,000 lux for 30 minutes; or (b) simultaneous exposure, ie, cells were concomitantly exposed to drug and light (50,000 lux) for 30 minutes.

In all experiments, a total of 10 × 10⁶ cells was treated at a final concentration of 5 × 10⁶ cells/mL in RPMI 1640 containing 1% FCS. This low FCS concentration was chosen for our studies since serum content has been previously shown to be inversely correlated to DHE and MC-540 uptake of cells.^{22,28} In BM-mixing studies, tumor cells were regularly incubated together with varying numbers of irradiated (4,500 rad) normal marrow cells. During drug and light exposure, cells were kept in suspension by swirling the test tubes gently every five minutes. After incubation, the cells were washed twice in RPMI 1640 and set up in colony-forming assays.

BM cells were resuspended in FCS and cultured in 35 × 10-mm Lux Petri dishes (Miles Laboratories, Naperville, IL). The CFU-GM, BFU-E, and CFU-GEMM assays were performed as described previously.²⁹ For CFU-GM, each cell culture consisted of 100,000 cells in 1.0 mL of McCoy's 5A medium containing 20% FCS, 10% Mo-T cell line-conditioned medium (kindly provided by Dr David Golde, UCLA), and methylcellulose (Methocel, A4M, Dow Chemical Co, Midland, MI) at a final concentration of 1.1%. Quadruplicate cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and cellular aggregates of more than 40 cells were counted on day 10. For BFU-E and CFU-GEMM, cell cultures consisted of 1 mL of Iscove's modified Dulbecco's medium (GIBCO) containing 25% FCS, 0.8% deionized bovine serum albumin (Sigma Chemical Co, St Louis), 10% of a selected lot of MoCM, 10⁻⁴ mol/L 2-mercaptoethanol (Sigma), 1 unit of partially purified human urinary erythropoietin (Toyobo, New York), and methylcellulose at a final concentration of 1.3%. Quadruplicate cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. On day 14, hemoglobinized cell aggregates of more than 50 cells were counted as BFU-E; mixed colonies were scored *in situ*, and their cellular composition was verified by removal and subsequent staining of selected colonies with May-Grünwald-Giemsa.

For statistical analysis of CFU-GM, BFU-E, and CFU-GEMM data, the *t* test of mean differences was performed wherever applicable.

Neoplastic cells, after incubation alone or in mixtures with irradiated normal marrow cells, were resuspended in FCS and cultured in 35 × 10-mm Petri dishes. Culture medium for each plate consisted of 1 mL of 1.1% methylcellulose containing 20% FCS, 20% RPMI 1640, 1% PSN, and 1% L-glutamine. Two hundred and 100 untreated cells or 10⁴ and 10³ treated cells per plate were cultured on 1-mL underlayers consisting of 0.5% agar, 20% FCS, and 1% PSN in McCoy's 5A medium. Quadruplicate cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and scored for cellular aggregates of more than 50 cells on day 8.

RESULTS

Clonogenic tumor cell reduction in response to photoradiation with DHE or MC-540. In combination with white light, both DHE and MC-540 showed a dose-dependent *in vitro* cytotoxic effect on neoplastic myeloid (HL-60) and lymphoid (Reh, SK-DHL-2) cells. After exposure to DHE at 2.0 to 2.5 µg/mL or to MC-540 at 15 to 20 µg/mL, clonogenic tumor cells from all three cell lines could be abolished beyond the limits of accurate detection (Fig 1). However, cell sensitivity towards photoradiation with DHE or MC-540 varied under different conditions of light activation. At the concentrations given, clonogenic reduction of HL-60 and SK-DHL-2 cells was markedly higher with DHE (>4 logs) than with MC-540 (1 to 2 logs) under conditions of sequential exposure whereby cell lines were drug incubated in the dark, washed, and subsequently exposed to white light at 50,000 lux; no such difference was observed after sequential treatment of Reh cells (Fig 1A and D). In contrast, simultaneous exposure of cells to drug and light resulted in the apparent total elimination of HL-60, Reh, and SK-DHL-2 cell colonies (>4-log reduction) after treatment with either DHE (2.0 µg/mL) or MC-540 (15 to 20 µg/mL) (Fig 1B and C). In all experiments, Reh cells were more sensitive toward DHE- and toward MC-540-mediated phototoxicity as compared with HL-60 and SK-DHL-2 cells. Incubation of cells in the dark with and without drug exposure yielded a mean recovery ± SD of 100.4% ± 3.1% and 100.2% ± 2.8% respectively when compared with drug-untreated control cells that were exposed to light only (the mean number ± SD of control colonies indicated as 100% recovery was 3,150.0 ± 115.1, 6,675.0 ± 463.1, and 4,108.4 ± 130.4 per 10⁴ cells for HL-60, Reh, and SK-DHL-2 respectively). As hereby indicated, cytotoxic effects from illumination-related hyperthermia could be ruled out.

Effect of photoradiation with DHE or MC-540 on various human hematopoietic progenitor cells (CFU-GM, BFU-E, CFU-GEMM). The recovery of CFU-GM was dependent on drug concentrations and on the modality of light activation. When BM mononuclear cells were sequentially treated, ie, preincubated with DHE in the dark and subsequently exposed to white light, colony growth was inhibited by 52.6% ± 7.6% and 70.7% ± 13.1% (mean ± SD) at DHE concentrations of 2.0 and 2.5 µg/mL respectively (ie, doses sufficient to induce approximately 4 logs of cell reduction in HL-60, Reh, and SK-DHL-2) (Fig 1A). In contrast, an apparent total reduction of CFU-GM colonies was observed after simultaneous drug and light exposure (2.0 µg/mL of DHE) of marrow cells (Fig 1C).

By using MC-540, a mean ± SD of 27.9% ± 8.6% and

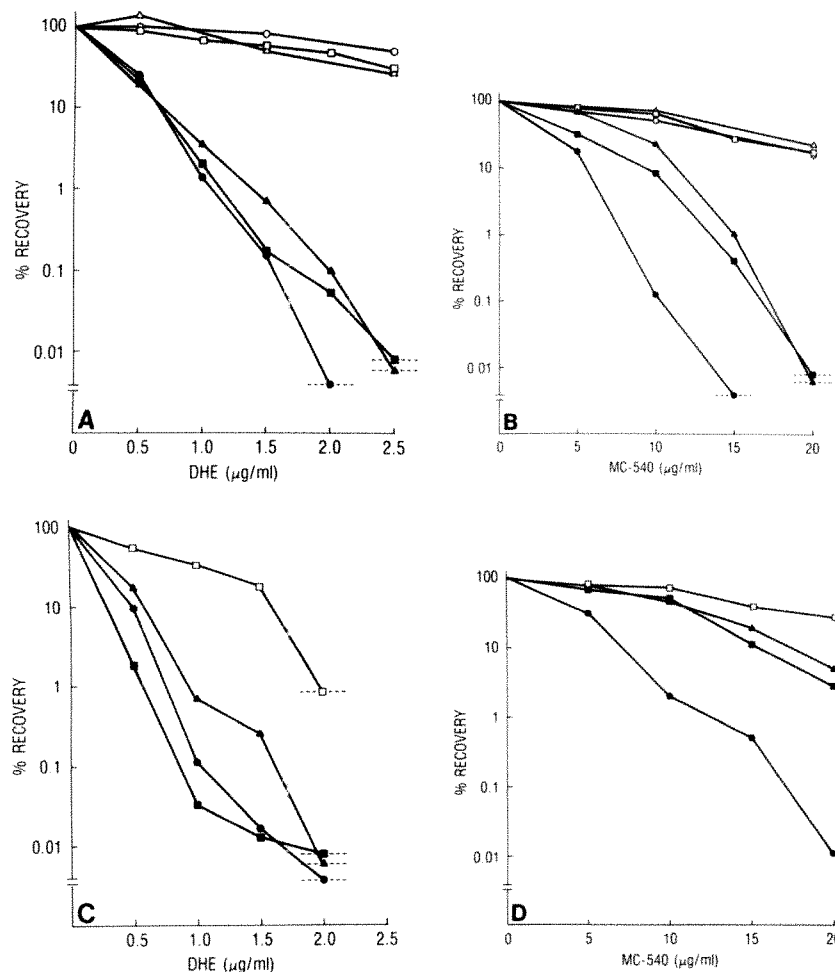


Fig 1. Photoradiation effects on normal human BM CFU-GM ($\square$), BFU-E ($\circ$), and CFU-GEMM (Δ) as compared with clonogenic growth reduction of HL-60 ($\blacksquare$), Reh ($\bullet$), and SK-DHL-2 ($\blacktriangle$) cells. (A) DHE/sequential drug-light treatment. (B) MC-540/simultaneous drug-light treatment. (C) DHE/simultaneous drug-light treatment. (D) MC-540/sequential drug-light treatment. Mean values from three or more separate experiments are given as the percentage of control (photoradiation in the absence of DHE or MC-540); ----- represents the lower limits of accurate detection as calculated from control experiments.

16.7% $\pm$ 4.8% CFU-GM was recovered after simultaneous light and drug treatment of BM mononuclear cells at dye concentrations of 15 and 20 $\mu\text{g/mL}$ respectively (ie, doses capable of abolishing approximately 4 logs of clonogenic HL-60, Reh, and SK-DHL-2 cells) (Fig 1B). The CFU-GM yield was 38.1% $\pm$ 3.4% and 26.1% $\pm$ 2.0% (mean $\pm$ SD) at the same doses of MC-540 (15 and 20 $\mu\text{g/mL}$ respectively) when drug incubation and light exposure of cells were done sequentially (Fig 1D). The mean clonogenic proliferation $\pm$ SD of control CFU-GM (BM cells were exposed to white light in the absence of DHE or MC-540) was 29.5 $\pm$ 6.7 colonies/ 10^5 cells. All CFU-GM data were statistically significant ($P \leq .05$).

Under conditions of selective photoradiation with DHE (sequential exposure) or MC-540 (simultaneous exposure), a dose-dependent clonogenic inhibition of erythroid progenitor cells (BFU-E) was noted (Fig 1A and B). A mean $\pm$ SD of 46.8% $\pm$ 8.4% BFU-E-derived colonies was recovered after treatment with DHE at 2.5 $\mu\text{g/mL}$. Exposure of normal marrow cells to MC-540 at 20 $\mu\text{g/mL}$ yielded 16.5% $\pm$ 2.7% BFU-E (mean $\pm$ SD), as calculated from photoradiated but drug-unexposed controls (mean clonogenic proliferation $\pm$ SD of 72.9 $\pm$ 11.2 erythroid colonies/ 10^5 cells). Results from BFU-E experiments were statistically significant ($P \leq .05$).

The assay system used for human erythroid progenitor

cells was also found to adequately support the growth of multipotent CFU-GEMM colonies (mean clonogenic proliferation $\pm$ SD of 4.6 $\pm$ 2.1 mixed colonies per 10^5 cells). At a DHE concentration of 2.5 $\mu\text{g/mL}$, a mean CFU-GEMM recovery $\pm$ SD of 27.5% $\pm$ 16.9% was observed after sequential drug and light exposure of BM mononuclear cells (Fig 1A). The CFU-GEMM yield was 20.1% $\pm$ 18.6% (mean $\pm$ SD) under selective conditions of MC-540-mediated phototoxicity, ie, simultaneous light and drug treatment of marrow cells at a dye concentration of 20 $\mu\text{g/mL}$ (Fig 1B). For CFU-GEMM data, the significance level was calculated at $P = .2$.

DHE- or MC-540-mediated phototoxicity against leukemia and lymphoma cells admixed with irradiated normal human BM. To simulate ex vivo BM-purging conditions, irradiated marrow cells were treated together with neoplastic myeloid (HL-60) and lymphoid (Reh, SK-DHL-2) cells. The clonogenic reduction of tumor cells in mixed populations was dose dependent and appeared to be slightly higher than with neoplastic cells alone (Fig 2). For DHE- and MC-540-treated cell populations, different modes of light activation were chosen to provide conditions of selective cytotoxicity against tumor cells (for comparison see Fig 1A and B). After sequential treatment with DHE and white light, the colony-forming capacity of all three neoplastic cell lines

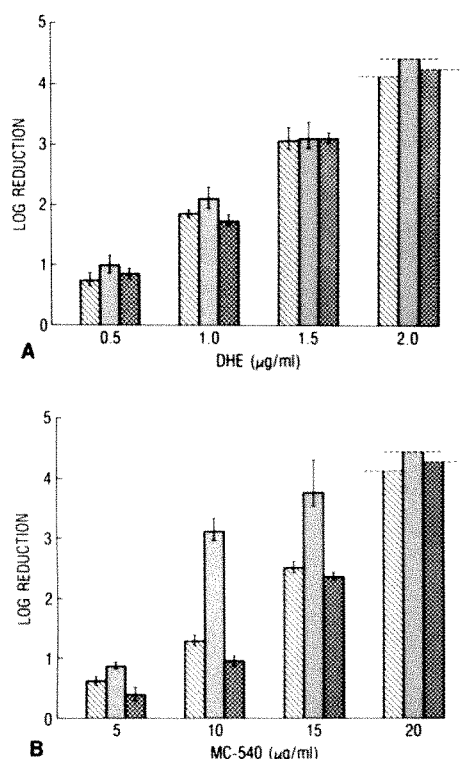


Fig 2. Phototoxicity-dependent clonogenic growth reduction of tumor cells in mixtures with irradiated normal human BM cells. Photoradiated (50,000 lux) cell mixtures were either preincubated with DHE in the dark (A) or simultaneously exposed to MC-540 (B). Mean logarithmic values ($\pm$ SD) represent a reduction in colony formation, as calculated from 100% recovery (photoradiation in the absence of DHE or MC-540). Combined photoradiation and drug treatment of cells with either 2.0 μ g/mL of DHE or 20 μ g/mL of MC-540 resulted in apparent total elimination of clonogenic tumor cells; ----- represents the lower limits of accurate detection as calculated from control experiments. □, HL-60; ▨, Reh; ▤, SK-DHL-2.

could be reduced by more than 4 logs at a drug dose of 2.0 μ g/mL (Fig 2A). Similar results were obtained when photoradiated cell mixtures were simultaneously exposed to MC-540 at 20 μ g/mL (Fig 2B). Under optimal conditions of photoradiation with DHE (2 μ g/mL, sequential exposure) or MC-540 (20 μ g/mL, simultaneous exposure), malignant clonogenic cells from all three cell lines (HL-60, Reh, and SK-DHL-2) could be abolished beyond the limits of detection when normal irradiated marrow and tumor cells were mixed at ratios of 1:1, 10:1, and 100:1 respectively (Table 1). However, differences in sensitivity were observed when comparing DHE- and MC-540-mediated phototoxicity. While the dose response to DHE was similar in all tumor cell lines tested (Fig 2A), MC-540 had a significantly higher tumoricidal effect on CALLA-positive, non-B, non-T, Reh cells as compared with HL-60 and SK-DHL-2 cell lines (Fig 2B).

DISCUSSION

The data presented confirm the preferential cytotoxicity of various light-activated compounds (DHE, MC-540) against neoplastic cells.^{1-8,17,18,24} The results from our study

Table 1. Comparison of DHE- and MC-540-Mediated Phototoxic Effects Against Leukemia and Lymphoma Cells Admixed With Different Ratios of Normal Irradiated Human BM Cells

Normal BM: Tumor Cell Mixing Ratio		Colonies per 10 ⁴ Tumor Cells		
		HL-60	Reh	SK-DHL-2
100:1	Control	2,608 $\pm$ 256	6,118 $\pm$ 477	3,775 $\pm$ 183
	DHE	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	MC-540	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
10:1	Control	2,912 $\pm$ 279	6,225 $\pm$ 766	3,938 $\pm$ 268
	DHE	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	MC-540	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
1:1	Control	3,070 $\pm$ 323	6,436 $\pm$ 584	4,163 $\pm$ 244
	DHE	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	MC-540	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0

Cells were sequentially (DHE, 2 μ g/mL) or simultaneously (MC-540, 20 μ g/mL) drug exposed and photoradiated (50,000 lux); control cells were photoradiated only. Values represent means ($\pm$ SD) of three separate experiments.

indicate the potential usefulness of photoradiation therapy for the clinical ex vivo treatment of tumor cell-contaminated BM grafts. Neoplastic cells from myeloid (HL-60) and lymphoid (Reh, SK-DHL-2) cell lines were highly susceptible (>4 logs of clonogenic growth reduction) to the tumoricidal effects of phototherapy with DHE or MC-540. Apparent total elimination of clonogenic tumor cells could be achieved at DHE concentrations of 2.0 to 2.5 μ g/mL and MC-540 doses of 15 to 20 μ g/mL respectively. Cytotoxicity against neoplastic cells was not affected when leukemia and lymphoma cells were treated in mixtures with irradiated normal human BM to simulate ex vivo purging conditions. However, differential sensitivity towards photoradiation treatment of neoplastic cells v normal hematopoietic precursors was highly dependent on the modality of light activation. When using DHE, a selective cytotoxic effect against tumor cells could only be observed under conditions of sequential drug and light exposure, whereas treatment with MC-540 yielded comparable results when cells were simultaneously drug treated and photoradiated with white light. Under these conditions, the more primitive, potentially engraftment-related CFU-GEMM precursors appeared to be less sensitive towards MC-540- but slightly more susceptible to DHE-mediated phototoxicity when compared with erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells.

Our findings reflect differences in the basic mechanisms of DHE- and MC-540-mediated cell photosensitization. Results from previous investigations show that both DHE and MC-540 preferentially localize in neoplastic cells.²⁰⁻²³ As reported, MC-540 incorporation can be four- to fivefold greater in leukemic cells than in normal human BM mononuclear cells.²² Similarly, DHE as well as other porphyrins accumulates in tumor cells rather than in normal tissue.²⁰ However, unlike MC-540, which virtually does not bind to cells in the dark,²² DHE is a permeant dye that accumulates in substantial amounts in the absence of light; furthermore, it is retained much longer in malignant than in most normal cells,^{20,21,30} a property that might be exploited by sequentially

exposing cells to DHE first and light second. For DHE, the cellular damage from photoradiation appears to be primarily mediated by the generation of reactive oxygen species such as singlet oxygen and hydroxyl radicals.³¹⁻³³ Differences in cell sensitivity towards MC-540, as reported in this and in previous studies, are most likely due to the electrical properties^{20,22,34-36} or the lipid composition³⁷⁻³⁹ of neoplastic cell membranes that allow the photoactive dye to preferentially damage these cells.

MC-540 has been used as an experimental BM-purging agent.^{18,24,40,41} In our study, we compared normal marrow and tumor cell clonogenicity in response to DHE and MC-540 under various conditions of light activation. In addition to CFU-GEMM and BFU-E, the CFU-GM assay was chosen as a reliable though limited screening method to develop purging conditions.⁴² Preservation of 27.5% and 20.1% of CFU-GEMM, 46.8% and 16.5% of BFU-E, and 29.3% and 16.7% of CFU-GM with highly tumoricidal doses (>4 log

reduction) of DHE and MC-540, respectively, supported a possible role for photoradiation therapy in clinical marrow-purging programs. In its preferential toxicity against a variety of neoplastic myeloid and lymphoid cells as compared with different normal hematopoietic progenitors, PRT appears to be similar to both pharmacological and immunologic BM purification regimens.^{9,11,14,43-49} However, given the various differences (eg, in cell proliferation and drug cytotoxicity) between clinical ex vivo and experimental in vitro purification of tumor cell-contaminated BM, further work will be needed to define the role of photoradiation therapy on its own or in combination with other methods⁵⁰ for the clinical ex vivo treatment of autologous BM grafts.

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An Inhibitory Monoclonal Antibody Against Human Tissue Factor

By Steven D. Carson, Sherman E. Ross, Ronald Bach, and Arabinda Guha

We obtained a hybridoma using immune spleen cells from a mouse injected with human brain tissue factor that had been purified on a factor VII-agarose affinity column. This monoclonal IgG1, HTF1-7B8, inhibits tissue factor procoagulant activity. The concentration of HTF1-7B8 producing half-maximal inhibition is influenced by the concentration of factor VIIa, suggesting that the antibody and enzyme compete for the cofactor. The antibody was successfully used to detect both human and bovine tissue factor on nitrocellulose dot blots, indicating that the epitope recognized by this antibody is conserved in both species. This

antibody clearly reveals tissue factor on a Western blot. An HTF1-7B8 affinity column was used to purify tissue factor from both human brain and placenta. The electrophoretic mobilities in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and the amino acid compositions of the purified tissue factor from brain and placenta are indistinguishable, as are their specific procoagulant activities in reconstituted systems. This antibody will be useful for immunopurification and characterization of tissue factor structure and mechanism.

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TISSUE FACTOR (coagulation factor III, tissue thromboplastin) is the membrane glycoprotein that serves as the nonenzymatic cofactor for factor VII in the initiation of blood coagulation. Although pure tissue factor has been available from bovine sources for several years,¹ human tissue factor has only recently been purified.^{2,3} This achievement was made possible by the discovery that immobilized human factor VII can serve as an affinity-matrix ligand for purification of detergent-solubilized tissue factor.^{2,3} Affinity chromatography using factor VII as the coupled ligand is a highly specific protocol, but tens of liters of human plasma are required as a source of the factor VII necessary to construct the affinity column. Insofar as the immunoaffinity purification of bovine tissue factor provides a practical avenue for purification of this protein,^{1,4} an antibody specific for human tissue factor is a highly desirable reagent. Using small quantities of human tissue factor purified by affinity chromatography on immobilized human factor VII, we successfully produced a monoclonal antibody that recognizes both brain and placental tissue factor.

MATERIALS AND METHODS

Coagulation factors VII and X were purified and prepared for use in these experiments as previously described.⁵⁻⁷ Bovine brain tissue factor was purified using monoclonal antibody TF1-F7,⁴ whereas human tissue factor was isolated using factor VII-Affi Gel.^{2,3} Tissue factor was reconstituted with phosphatidylserine and phosphatidylcholine (30/70) (Supelco, Bellefonte, PA) using octylglucoside followed by dialysis⁸ or with mixed brain lipids using the deoxycholate-CdCl₂ protocol.^{9,10} Tissue factor activity was measured with a two-stage clotting assay^{8,11} or a continuous chromogenic rate assay,^{12,13} using bovine factors VIIa and X.

Plasmacytoma cells P3-NSI-1-Ag4-1 (NS-1) were cultured as

previously described,¹⁴ and NCTC 109 was included at 5% in the HY media. The following immunization protocol provided hyperimmune spleen cells from a single mouse, which were used to obtain hybridomas.¹⁵ Human brain tissue factor (HBTF), 20 µg, was combined with 1 µg of cholera toxin (from Dr Alex Kurosky, University of Texas Medical Branch at Galveston), 210 µL of 0.9% NaCl and 200 µL of Freund's complete adjuvant (GIBCO, Grand Island, NY). The mouse received the entire inoculum intraperitoneally (IP) on day 1. Three weeks later, the animal received an IP booster containing 13 µg of HBTF in 200 µL of saline and 200 µL of Freund's incomplete adjuvant (GIBCO). On day 39, 7 µg of HBTF was administered intravenously (IV) followed by another IV boost of 7 µg on day 57. On day 60, the mouse was killed, and cells harvested from the spleen were fused with NS-1 cells using polyethylene glycol (PEG) 1540 (American Type Culture Collection).¹⁴ Cells were plated and grown in selective medium as previously described.¹⁴

Culture supernatants were screened for antibodies recognizing TF using a multistep enzyme-linked immunosorbent assay (ELISA) similar to the one developed for use with bovine TF.⁴ The wells of an Immulon II microtiter plate were sequentially incubated (interspersed with washes) with rabbit antibodies against mouse immunoglobulins (DAKO, Santa Barbara, CA), spent culture media from the supernatants to be tested, Tris-saline (0.05 mol/L of Tris, 0.1 mol/L of NaCl, 0.01% NaN₃, pH 7.6) containing 10 mg/mL of bovine serum albumin (BSA), biotinyl-HBTF, and avidin-horseradish peroxidase conjugate. Bound peroxidase activity was detected using phenylenediamine.¹⁶ The biotinyl-HBTF used was HBTF, oxidized with sodium metaperiodate, reacted with biotin-hydrazide (Sigma, St Louis), and reduced with sodium cyanoborohydride.^{17,18} Avidin was conjugated to horseradish peroxidase (Sigma, type VI) using the two-step glutaraldehyde method.¹⁶ Cultures that produced positive ELISA results (visible peroxidase product in the well) were expanded, cloned, and frozen.^{4,14}

Tissue factor on nitrocellulose (Schleicher and Schuell, BA85) either as "dot blots" or Western blots¹⁹ was detected with HTF1-7B8. After TF was bound, the nitrocellulose was blocked with 3% gelatin in Tris-saline. The blot was incubated overnight at room temperature with 1% gelatin, 0.05% Tween 20, and HTF1-7B8 (0.4 µg/mL) in Tris-saline. Following washes with 0.05% Tween 20 in Tris-saline, the blot was incubated with horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (Bio-Rad, Richmond, CA, 1/2,000) in 1% gelatin, 0.05% Tween 20, Tris-saline for 2 hours. After further washing, the bound antibodies were developed using 4-chloro-1-naphthol.²⁰ No staining was observed when HTF1-7B8 was omitted from the sequence.

The capacity of HTF1-7B8 IgG to inhibit TF activity was ascertained using both coagulation and chromogenic assays. Increasing amounts of HTF1-7B8 IgG or a control IgG (RIg9C¹⁴) were added to a constant amount of human brain homogenate used as a TF "standard."¹¹ Activity was then quantitated in the presence of

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factor VIIa and factor X, using concentrations as described in the Results section. Curves were fit and equation parameters were estimated using the direct-plot method.²¹

Tissue factor was immunoaffinity purified using 22 mg of HTF1-7B8 IgG coupled to 50 mL of Affi-Gel 10 (Bio-Rad). Acetone powders of human placenta and brain were prepared and extracted with Tris-saline, 0.1% Triton X-100, and finally with 2% Triton X-100 essentially as reported,^{1,11,22} using 10 mL of solution per gram of powder. Approximately 150 mL of extract was applied to the IgG-Affi-Gel, which was then washed with 5 column vol of 0.1% Triton X-100 in Tris-saline followed by 0.1% Triton X-100 in Tris-saline with 0.9 mol/L of NaCl. The TF was eluted with 0.1 mol/L of glycine, 0.1 mol/L of NaCl, 0.1% Triton X-100, pH 2.^{1,4} Gel filtration on Ultragel AcA 44 (LKB) in Tris-saline with 0.1% Triton X-100 removed minor high-mol-wt proteins, providing TF that was homogeneous on polyacrylamide gels. The immunoaffinity column has been used repeatedly over a period of 6 months with no apparent loss of effectiveness. The amino acid compositions of the TF preparations were obtained by the Yale Protein Chemistry Facility following 16-hour hydrolysis in 6 N HCl containing 0.2% phenol at 115°C.

RESULTS

Of the cultures that produced antibodies against HBTF as indicated by the ELISA, the 7B8 culture was most consistently positive, rapidly growing, and stable after cloning. Immunodiffusion against chain type-specific antibodies indicated that this hybridoma produced an IgG with γ_1 and κ chains. This hybridoma, designated HTF1-7B8, produced ample ascites when grown in pristane-primed mice, and the IgG was easily purified by ammonium sulfate precipitation, chromatography on diethylaminoethyl (DEAE) Sephacel and, as required, gel filtration on Sephacryl S-300 to remove transferrin.^{23,24}

Having shown that HTF1-7B8 bound biotinyl-TF in the ELISA, we tested the purified IgG for effects on the activity of TF reconstituted with phospholipids. This antibody suppressed TF activity in both clotting and continuously monitored chromogenic assays. Kinetic effects of HTF1-7B8 IgG were ascertained using the chromogenic assay, with a 1/12,000 dilution of human brain homogenate used as "standard" tissue factor,¹¹ and varying concentrations of both the antibody and factor VIIa. As shown in Fig 1, TF activity was inhibited by added HTF1-7B8 IgG, and inhibition approached 100% as antibody exceeded 30 nmol/L. As shown in the inset in Fig 1, the maximum attainable inhibition was not affected by the concentration of factor VIIa, but the concentration of HTF1-7B8 IgG required to achieve half-maximal inhibition decreased as the factor VIIa concentration was reduced to <0.5 nmol/L.

HTF1-7B8 also recognized TF antigen on nitrocellulose. HBTF, purified by affinity chromatography on factor VII-Affi-Gel,³ was subjected to electrophoresis in a 10% polyacrylamide gel after reduction and denaturation in sodium dodecyl sulfate (SDS).²⁵ The TF, electrophoretically transferred onto nitrocellulose, was immunochemically detected with HTF1-7B8 IgG (Fig 2). As in previous reports,^{2,4} an antigen with electrophoretic mobility somewhat greater than that of the phosphorylase b mol-wt standard was also demonstrable. In "dot" blots, <1 ng of human TF was clearly

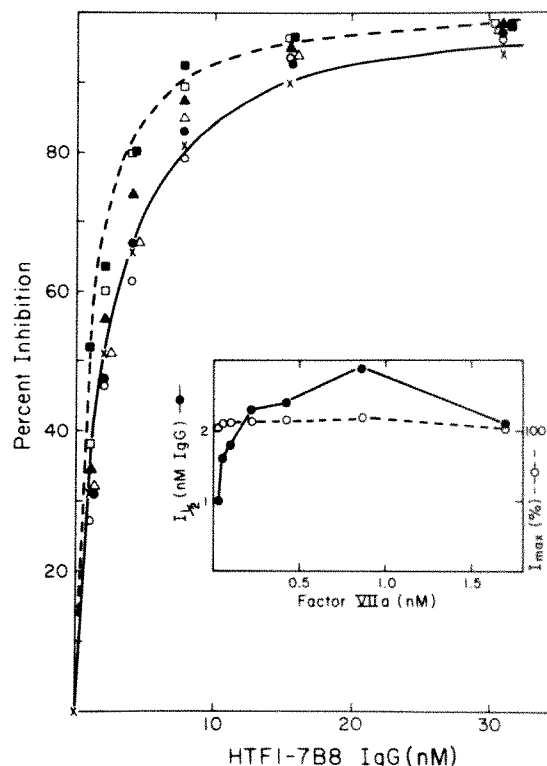


Fig 1. Inhibition of human brain tissue factor procoagulant activity by HTF1-7B8 IgG in the continuous chromogenic rate assay. Factor X was present in all assays at 100 nmol/L. Factor VIIa concentrations ranged from 26.7 pmol/L (■), to 1.71 nmol/L (X), with direct-plot fitted curves as indicated (—■— and —X—), respectively. Inset illustrates the effect of factor VIIa concentration on the maximum attainable inhibition (—○—) and on the concentration of HTF1-7B8 IgG required to achieve half-maximal (—●—) inhibition of factor X activation.

detectable by the immunochemical staining (Fig 3). This experiment also revealed immunologic reactivity of HTF1-7B8 IgG with bovine brain TF with an empirically estimated fourfold decrease in sensitivity relative to the reactivity with HBTF (Fig 3).

HTF1-7B8 IgG, coupled to Affi-Gel 10, was used to isolate TF factor from 2% Triton X-100 extracts of human brain and placenta. The immunoaffinity matrix routinely removed all detectable TF activity from the extracts, and homogeneous TF protein was recovered from the affinity matrix. From 150 mL of 2% Triton X-100 extract, 140 to 150 μ g of tissue factor was routinely obtained, representing an estimated recovery of 75%. The 10% polyacrylamide gel analysis of the brain and placental TF isolates confirmed their electrophoretic similarities (Fig 4).²⁶ This comparison also established that the immunoaffinity and factor VII affinity-purified tissue factors were electrophoretically indistinguishable. When reconstituted into lipid vesicles, the brain and placental tissue factor isolates exhibited similar specific activities in the clotting assay. One picogram of bovine brain TF, reconstituted into PS/PC (30/70) vesicles was defined as one unit.^{1,8} This purified and reconstituted TF provided a log-log calibration curve of units v clot time for

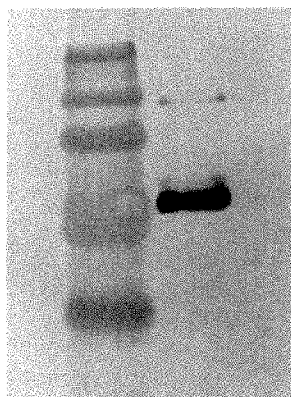


Fig 2. Western blot of factor VII affinity-purified human brain tissue factor using HTF1-7B8 as the immunochemical probe. The bands in the lane farthest left are the prestained electrophoretic standards (BRL), from top to bottom, myosin H chain (mol wt 200,000), phosphorylase b (mol wt 97,400), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), α -chymotrypsinogen (mol wt 25,700), β -lactoglobulin (mol wt 18,400), and lysozyme (mol wt 14,300).

the two-stage coagulation assay which was linear from 1,000 to 125 U/mL. When reconstituted into mixed brain lipids, both human brain and placental TF had specific activities of 0.8 U/pg. Both brain and placental TF reconstituted into PS/PC (30/70) using octylglucoside had specific activities of 1.0 U/pg.

In further investigation of their similarities, the TF apoproteins from brain and placenta were subjected to acid hydrolysis and amino acid analysis. As shown in Table 1, brain and placental TF have indistinguishable amino acid compositions.

DISCUSSION

An IgG₁ monoclonal antibody has been produced that clearly recognizes human tissue factor. Unlike previous claims,^{27,28} this monoclonal antibody binds human TF by multiple criteria. The antibody bound pure biotinyl-TF from solution in the ELISA screening assay. Conversely, pure TF immobilized on nitrocellulose bound HTF1-7B8 IgG from solution in both Western and dot-blot procedures. Significantly, HTF1-7B8 IgG immobilized on Affi-Gel 10 selec-

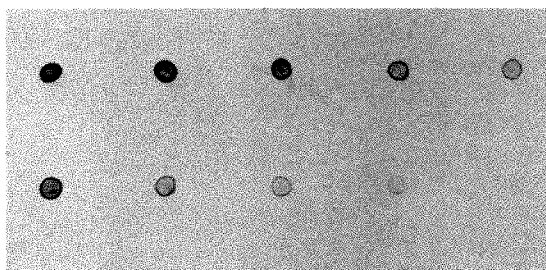


Fig 3. Immunoblot detection of brain tissue factor (TF) on a nitrocellulose dot-blot using HTF1-7B8. The upper row was spotted with human brain TF, and the lower row was spotted with bovine brain TF. From left, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, and 0.05 ng of protein were spotted in each dot in 1 μ L of Tris-saline with 0.1% Triton X-100.

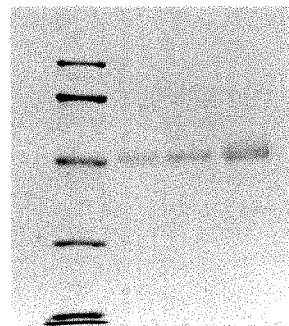


Fig 4. Coomassie R-250-stained polyacrylamide gel (10%) following electrophoresis of purified tissue factor (TF) with sodium dodecyl sulfate (after reduction). The mol-wt standards (left lane) were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase (mol wt 31,000), soybean trypsin inhibitor (mol wt 21,500), and lysozyme. The other three lanes contained (left to right) 3.9 μ g of factor VII affinity-purified human brain TF, 4.5 μ g of HTF1-7B8 affinity-purified human brain TF, and 5.6 μ g of HTF1-7B8-affinity-purified human placental TF.

tively bound TF from among the myriad proteins of the Triton X-100 extract,²⁶ and enabled immunoaffinity purification of the apoprotein. Furthermore, and of least significance in proving the antibody specificity for TF, HTF1-7B8 IgG inhibits TF activity. In the aggregate, these results present compelling evidence for the specific recognition of TF by this monoclonal antibody.

Using immunoaffinity purification, sufficient quantities of human brain and placental TF have been isolated for partial characterization. Among the first direct results of these experiments, the antibody clearly confirmed the immunologic similarities of human brain and placental TF,²⁶ and demonstrated partial immunological similarity between human and bovine TF proteins. The electrophoretic mobilities of apoproteins isolated from brain and placenta were the

Table 1. Amino Acid Composition of Tissue Factor

Amino Acid	Composition (mol %)		
	Brain (2 Analyses)	Placenta (4 Analyses)	Broze and Colleagues ²
Asx	10.48	10.93	11.50
Thr	9.00	9.77	9.28
Ser	7.95	7.24	8.28
Glx	11.77	11.93	11.65
Pro	4.99	4.81	4.48
Gly	7.83	6.49	7.75
Ala	5.13	5.09	5.05
$\frac{1}{2}$ Cys	ND	ND	0.78
Val	8.30	8.80	8.05
Met	0.79	0.64	0.80
Ile	4.25	4.26	3.95
Leu	7.39	7.56	6.90
Tyr	4.67	4.73	3.98
Phe	4.52	4.68	4.68
His	1.06	0.97	0.95
Lys	7.45	7.74	7.58
Arg	4.44	4.37	4.30
Trp	ND	ND	ND

Abbreviation: ND, not determined.

same on polyacrylamide gels in SDS, consistent with previous conclusions based solely on eluted and reconstituted activity.²⁶ Furthermore, the immunoaffinity purified and factor VII-affinity purified materials were indistinguishable. On the Western blot, brain TF apoprotein was localized at a position consistent with an estimated mol wt of 47,500, which is larger than bovine TF.^{1,3,4} This conclusion is consistent with results of the polyacrylamide gel analysis, where brain and placental TF comigrate. The mol wt, estimated for reduced apoproteins, is less than that previously estimated using activity recovered from polyacrylamide gradient gels without prior reduction.²⁶ The high-mol-wt band detected near 96,000 mol wt on the Western blot remains to be characterized. A protein of similar apparent mol wt has been observed in preparations of factor VII-affinity purified human TF.^{2,3} A protein of 85,000 mol wt has also been

observed in preparations of bovine TF and has been suggested to be a dimeric form of the apoprotein.^{1,4} Contrary to one preliminary report,²⁹ the human brain and placental TF isolated in this study have similar specific activities in the two-stage coagulation assay whether relipidated using octylglucoside⁸ or CdCl₂ and deoxycholate⁹ reconstitution protocols. Finally, the amino acid compositions of brain and placental TF proteins are indistinguishable from one another, and are not notably different from the previously published amino acid composition of factor VII-purified HBTF.² From these data TF apoproteins from human brain and placenta appear to be essentially identical.

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Lymphocyte Interleukin 2 Production and Responsiveness Are Altered in Patients With Primary Myelodysplastic Syndrome

By Olcay Ayanlar-Batuman, Jerry Shevitz, Ursula C. Traub, Scott Murphy, and Daniel Sajewski

Immunoregulatory T and B cell functions in 15 patients with primary myelodysplastic syndrome (MDS) were studied by measuring the proliferative and the stimulatory capacity of T and B cells, respectively, in autologous (auto) and allogeneic (allo) mixed lymphocyte reaction (MLR). T cell proliferation in the auto MLR was 25% of the control ($P < .02$), whereas proliferation in the allo MLR was normal. When control T cells were stimulated by MDS B cells, their proliferative response was only 57% of the control ($P < .01$). The mechanism responsible for these abnormalities was studied by determining the capacity of MDS and normal T cells to produce interleukin 2 (IL 2) and to generate IL 2 receptors (IL 2R) following stimulation with control and MDS B cells. In the auto MLR of MDS patients, only $3\% \pm 2\%$ of T cells developed IL 2R positivity, whereas in control cultures $12\% \pm 2\%$ of T cells were positive, as determined by immunofluorescence, using a monoclonal antibody (MoAb) directed against the IL 2R, and FACS analysis. When MDS T cells were stimulated by control B

cells, IL 2R generation and the production of IL 2 were within normal limits. In contrast, when control T cells were stimulated by MDS B cells or control B cells, the MDS B cells induced production of only 26% of IL 2 as compared with control B cells. In parallel experiments, IL 2R generation in control T cells stimulated by either MDS or control B cells was similar. We conclude that in the primary MDS, T and B cell interactions are impaired. Although MDS T cells develop normal quantities of IL 2R and produce normal amounts of IL 2 when stimulated by control B cells, they are markedly impaired when stimulated by self B cells. Similarly, MDS B cells can induce IL 2R generation in control T cells but not in MDS T cells. Myelodysplastic B cells are also defective in inducing IL 2 production by normal T cells in an allo MLR. These in vitro abnormalities strongly suggest that generation of lymphocytes with immunoregulatory functions is impaired in patients with MDS.

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THE PRIMARY myelodysplastic syndrome (MDS) consists of a heterogeneous group of bone marrow disorders characterized by ineffective and dysplastic hemopoiesis in one or more cell lines.¹ The basic abnormality in this syndrome is a clonal disorder of the hemopoietic stem cells.²⁻⁴ In 20% to 75% of cases, MDS evolves to acute nonlymphocytic leukemia.^{5,6} Recent evidence suggests that lymphocytes of MDS patients are derived from the same clone as the abnormal erythroid and myeloid cells.⁷ There is often a peripheral blood lymphopenia with^{8,9} or without^{10,11} T cell subset distribution abnormalities. T cells may show increased radiosensitivity, reduced proliferative response to mitogens, and poor colony formation in vitro.^{10,12} B cells may be deficient in Epstein-Barr virus (EBV) receptors.¹³ Natural killer (NK) cell functions may be abnormal, and their numbers may be reduced.^{14,15} Although phenotypic and functional abnormalities of T cells and NK cells are reported, immunoregulatory mechanisms in MDS have not been studied.

Alterations in immunoregulatory T cell functions and in lymphokine production, especially interleukin 2 (IL 2) are observed in patients with acute lymphoblastic leukemia and aplastic anemia.¹⁶⁻¹⁸ Because T cells contribute to the regulation of hemopoiesis, abnormalities in their numbers and functions are believed to play a pathophysiologic role in these hematologic dyscrasias.^{19,21} To determine the immunoregulatory lymphocyte functions in MDS, 15 patients were studied

for the proliferative and stimulatory functions of T and B cells, respectively, as well as production of IL 2 and generation of IL 2 receptors (IL 2R) in an autologous (auto) or allogeneic (allo) MLR.

MATERIALS AND METHODS

Patients. Fifteen patients (13 males and two females) were studied (Table 1). Diagnosis of primary MDS was confirmed by morphological study of bone marrow aspirates and peripheral smears stained with Wright's Giemsa. The percentage of ring sideroblasts was determined by prussian blue reaction for iron. The French-American-British (FAB) group criteria were used for diagnosis and classification of each patient.¹ Patients with refractory anemia or refractory anemia with ring sideroblasts had $<5\%$ blasts in the bone marrow. Patients with refractory anemia with excess blasts had $<5\%$ blasts in the peripheral blood and 5% to 20% blasts, which did not contain Auer rods, in the bone marrow. None of the patients had received cytotoxic agents or corticosteroids within the 6 months prior to the study of their lymphocytes. Five patients had not received transfusion with packed RBC (PRBC) transfusions. Ten patients were transfused; of these, 8 received 10 to 312 U and 2 had <10 U of PRBC. Controls were healthy, age-matched volunteers.

Isolation of lymphocytes and lymphocyte subpopulations. Peripheral blood lymphocytes were separated from heparinized whole blood by Ficoll-Hypaque centrifugation (Lymphocyte separation medium, Bionetics Laboratory Products, Litton Bionetics, Kensington, MD).²² Unfractionated mononuclear cells were depleted of macrophages by adherence to plastic. Nonadherent, mononuclear cells were separated into E rosette-positive (E+) ie, T cells, and E rosette-negative (E-) populations with neuraminidase (Sigma Chemical, St Louis) treated 5% sheep RBCs (SRBCs) (GIBCO Laboratories, Madison, WI).²³ The E+ T cell population was $<1\%$ surface immunoglobulin (Sig) positive, and $<3\%$ α -naphthyl-acetate-esterase (ANAE) positive, 97% formed re-rosettes with SRBCs and $95\% \pm 2\%$ reacted with anti-Leu 5 monoclonal antibody (MoAb) (Becton Dickinson, Immunocytometry Systems, Mountain View, CA) that reacts with the receptor for SRBCs.²⁴ Unrosetted cells at the Ficoll-medium interface are referred to as B cells. Of these, 96% were Sig+, $<1\%$ were E+ and $<3\%$ were ANAE positive. Viability of both E+ and E- populations was determined

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Table 1. Clinical Data of Patients With Primary MDS

Patient	Age	Diagnosis	Hemoglobin (g/dL)	WBC ($\times 10^3/\mu\text{L}$)	Platelets (μL)	Karyotype	Time From Diagnosis (mo)	RBC Transfusions Total (U PRBCs)
1. A.P.	74	RA	7.3	7.0	478,000	46XY, 5q-	48	11
2. O.S.	75	RA	9.9	3.0	198,000	47XY, +8	48	0
3. L.K.	75	RA	13.1	7.4	121,000	46XY	7	3
4. W.H.	64	RA	8.2	4.1	166,000	46XY	2	10
5. S.H.	76	RA	8.7	12.7	760,000	46XY	18	0
6. P.S.	58	RA	11.6	3.6	53,000	ND	12	10
7. F.S.	59	RA	12.3	4.9	238,000	ND	48	0
8. W.N.	70	RARS	9.0	2.3	65,000	ND	48	312
9. J.H.	56	RARS	7.3	5.0	28,000	46XY	48	10
10. E.S.	65	RAEB	8.4	2.2	32,000	ND	12	36
11. S.S.	74	RAEB	12.5	4.1	97,000	ND	18	13
12. M.M.	80	RA	7.0	3.0	78,000	ND	108	36
13. J.R.	63	RAEB	8.6	1.9	24,000	ND	1	0
14. J.B.	73	RAEB	8.1	4.3	39,000	53XY, -20, +5 +8, +10, +14, +22, +MI	ND	6
15. J.F.	80	RAEB	9.0	2.6	73,000	ND	1	0

Abbreviations: MDS, myelodysplastic syndrome; PRBCs, packed RBCs; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess blasts; ND, not done.

by exclusion of 0.05% (vol/vol) trypan blue in phosphate-buffered saline (PBS) and was >95%.

Cell surface phenotype analysis using immunofluorescent staining with MoAb. The following MoAbs were used to identify the lymphocyte populations and subsets: anti-Leu 5 (CD2), mature T cells; anti-Leu 2 (CD8), cytotoxic/suppressor T cells (Becton Dickinson)²⁵; anti-Leu 3 (CD4), inducer/helper T cells;²⁵ anti B4 (CD 19) (Coulter Immunology, Hialeah, FL), B cells²⁶; anti-HLA-DR (nonpolymorphic) (Becton Dickinson) is directed against the class II major histocompatibility (MHC) antigen complex and identifies B lymphocytes, monocytes/macrophages, and activated T cells²⁷; anti-IL 2 receptor (IL 2R) (CD25) (Becton Dickinson), activated T cells²⁸; anti-Leu 7 (Becton Dickinson), and NK cells.²⁹ Cell surface markers were determined by indirect immunofluorescence, applying first the relevant monoclonal antibody and then fluorescein conjugated (FITC) goat anti-mouse IgG (GAMIGG). Control cells were incubated with isotype-matched mouse IgG and FITC-GAMIGG. Cell surface fluorescence was determined by flow cytometry. The coexistence of a pair of cell surface antigens by dual fluorescence was determined by direct immunofluorescent method, applying first a FITC-labeled MoAb followed by a phycoerythrin (PE)-labeled MoAb to the cells. In all studies with two-color staining, the percentage positive for each surface marker was confirmed by single-color analysis. Control cells were incubated with isotype-matched FITC-mouse IgG and PE-mouse IgG.

Flow cytometry analysis. Flow cytometry analysis of cells stained by either indirect (single-color) or direct (two-color) immunofluorescence was performed using a FACS analyzer (Becton Dickinson). The percentage positive refers to the percentage of 25,000 lymphocyte-sized cells with more intense fluorescence than 99% of the background determined by incubation of lymphocytes with isotype-matched mouse IgG followed by staining with FITC-GAMIGG and/or PE-GAMIGG. Results were analyzed by a Consort 30 Data Management System (Hewlett Packard, Co).

The Auto and Allo MLR. For the auto MLR, responder T cells and stimulator B cells were obtained sterilely from a single individual. For the allo MLR, T cells and B cells were obtained from unrelated individuals. B cells to be used as stimulator cells were treated with mitomycin C (66 ng/mL) (Sigma). Responder and stimulator cells were cultured in RPMI, supplemented with 10%

heat-inactivated pooled AB serum, 10 mmol/L of HEPES (GIBCO, Grand Island, NY), 2 mmol/L of L-glutamine (GIBCO), and antibiotics (complete medium) in flat-bottomed microtest tissue culture plates (No. 3040 Falcon Labware, Oxnard, CA) for 6 days at 37°C in a humidified air atmosphere of 5% CO₂. Eighteen hours before harvesting 1 μCi of tritiated thymidine (³HTdR) (2 Ci/mmol, Schwarz/Mann, Spring Valley, NY) was added to each well. Cultures were performed in triplicate with controls consisting of T cells and of stimulator cells by themselves. Each patient was tested for MLR twice, and the mean cpm was used in the statistical analysis of the results.

To determine the generation of IL 2 receptors on T cells activated with mitogen or in an auto or an allo MLR, T cells were cultured and prepared for immunofluorescent staining and flow cytometric analysis as described above. Cell viability in these cultures was >85%.

IL 2 production and assay. T cells ($1 \times 10^6/\text{mL}$) were incubated with 2 $\mu\text{g}/\text{mL}$ of phytohemagglutinin (PHA-P, Burroughs Wellcome, Greenville, NC) in a humidified air atmosphere of 5% CO₂ for 48 hours in complete medium supplemented with 2% human AB serum (heat inactivated). Cell viability after culture was >95%. The supernatant was collected and filtered through a 0.45- μm filter (Nalgene Labware, Nalge Co., Rochester, NY) before storage at -70°C. T cells were also stimulated in an allo MLR, and IL 2 production was measured. In brief, responder and stimulator cells (0.1 mL of each) were cocultured in flat-bottomed tissue culture plates, as described for MLR cultures. After 36 hours of incubation, the contents of five wells were pooled, spun at 400 g for 25 minutes, and the supernatant stored at -70°C. Preliminary experiments showed the optimum time for collecting IL 2-containing supernatants in an allo MLR to be 36 hours. The IL 2 concentration in the supernatants was measured using a cytotoxic T lymphocyte (CTLL) assay, as previously described.³⁰ The IL 2 standard, a 1:16 dilution of rat IL 2 that was the supernatant obtained from spleen lymphocytes ($10 \times 10^6/\text{mL}$) incubated with 5 $\mu\text{g}/\text{mL}$ of concanavalin A (Con A) for 48 hours, was denoted as 1 U/mL.

Response to IL 2. Both auto and allo MLRs were cultured in medium alone or medium supplemented 1:5 (vol/vol) with various concentrations of recombinant human IL 2 (Amgen Biologicals, Thousand Oaks, CA). Fifty microliters of recombinant IL 2 (0.25 U/mL) was added to the cultures.

Statistical analysis. All statistical analysis was performed using Bell Laboratories' "S" statistical software program and a VAX-50 computer. Student's *t* test was used to determine the significance of the difference between experimental results obtained from patients and controls.

RESULTS

Surface characteristics and quantitation of lymphocyte subsets. Table 2 summarizes the surface reactivity of lymphocytes of MDS patients with MoAbs and compares them with those from age-matched healthy controls. Absolute numbers of total lymphocytes, T cells, and B cells were significantly diminished in all patients. Both CD4+ and CD8+ T cells were decreased to a similar extent (53.6% and 57.3% of control, respectively), keeping the helper/suppressor T cell ratio in the peripheral blood within normal limits, ie, 1.75. B cells, as assessed by the presence of the CD19 antigen, were comparable in percentage but significantly diminished in numbers, ie, 51.9% of control. In addition, the total numbers and percentage of NK cells were significantly diminished in all patients. Percentage of distribution and the absolute number of monocytes/macrophages determined by surface HLA-DR expression were normal. Both B cells and macrophages express HLA-DR antigens; therefore, the peripheral blood lymphocytes were simultaneously stained with FITC-labeled anti-HLA-DR and PE-labeled anti-CD19 antibodies and only the HLA-DR+ cells that did not express CD19 antigen were counted as macrophages.

Lymphocyte distribution in the peripheral blood varies with time. To determine the consistency of our findings, we performed the peripheral blood lymphocyte analysis on three occasions in four patients at 3-week intervals. We did not observe a significant variation in lymphocyte numbers or subset distribution. Changes in lymphocyte subset distribution also occur after multiple transfusions with blood products.^{31,32} Although 8 of 15 patients required frequent transfusions with RBCs (Table 1), no correlation was found between lymphopenia (total lymphocyte, T and B cells numbers) and the number of RBC transfusions in these patients.

Mixed lymphocyte reaction. To determine T cell functions in patients with MDS, we first investigated the T cell proliferative response to stimulation with autologous B cells in the auto MLR. T cells proliferate when cocultured with autologous non-T cells; this phenomenon is termed auto

MLR.³³ Class II MHC antigens expressed by the non-T cells play an essential role in activation of responding T cells in this reaction. Thus, HLA-DR antigens from stimulator cells render resting T cells sensitive to IL 2 and induce production of IL 2, which engages with its high-affinity receptor on the activated T cells, resulting in their proliferation. The auto MLR has specificity and memory,³⁴ and generates helper, suppressor, and cytotoxic T cells.³⁵⁻³⁷ In the patient population, the mean T cell proliferation in the auto MLR was significantly lower than control values ($P < .0002$) (Fig 1). To determine if MDS B cells were defective as stimulatory cells in the auto MLR, normal B cells were also used to stimulate MDS T cells. MDS T cells proliferated less than did control T cells in the allo MLR, but this difference was not statistically significant (Fig 2). To determine if an impairment in the stimulatory B cell function played a role in the low auto MLR, an allo MLR was performed using control T cells as responders and MDS B cells as stimulators. As shown in Fig 2, the MDS B cells induced a T cell proliferation that was 57% of that induced by control allogeneic B cells ($P < .01$). These findings showed that MDS B cells were impaired in stimulating autologous or allogeneic T cells to proliferate in a MLR and suggested the presence of defective T and B cell interactions in patients with MDS.

To determine the mechanism of low auto and allo MLR proliferation, we measured the IL 2 production and receptor generation in MLR-stimulated T cells of MDS patients. The amount of IL 2 generated in control and patient auto MLR was below the sensitivity of the CTLL assay. Therefore, we determined the capacity of T cells to produce IL 2 on activation with phytohemagglutinin (PHA) or with allogeneic B cells. IL 2 production by MDS T cells was normal when these cells were stimulated by either control B cells in an allo MLR or by lectin (Table 3). In contrast, when patients' B cells were used as stimulators, control T cells produced only 26% of the IL 2 produced after their stimulation with control allogeneic B cells. These results show that T cells from MDS patients can produce IL 2 normally when stimulated with mitogen or by control B cells; however, MDS B cells are defective stimulators of IL 2 production of both MDS and control T cells. One possibility that could be responsible for the defective B cell stimulatory activities in MDS is a decreased expression of cell surface DR antigen. The fluorescence intensity of DR antigen on the B cell surface was not different in patients and controls (Fig 3).

Table 2. Immunofluorescence Analysis of Lymphocyte Populations in MDS

	Number of PBLs (/μL)	Surface Antigen + (Absolute Numbers/μL)					
		CD2	CD4	CD8	CD19	Leu 7	HLA-DR
MDS (15)	1,457 ± 166*	929 ± 140*	545 ± 89*	305 ± 57*	120 ± 40*	150 ± 48*	611 ± 21
C (15)	2,011 ± 149	1,584 ± 170	1,015 ± 65	532 ± 60	231 ± 20	440 ± 97	457 ± 72
		Surface Antigen + (% positive)					
		CD2	CD4	CD8	CD19	Leu 7	HLA-DR
MDS (15)		67 ± 4	42 ± 4	24 ± 3	7 ± 1	9.6 ± 3*	30 ± 6
C (15)		70 ± 2.3	49 ± 1.3	25 ± 1.7	10 ± 0.8	16 ± 2.1	20 ± 2.2

Results are mean ± SEM; number of persons studied is given in parentheses.

Abbreviations: C, controls; PEL, peripheral blood lymphocytes.

*Significant difference ($P < .02$) on the basis of 95% of confidence limits between patients and normal control groups.

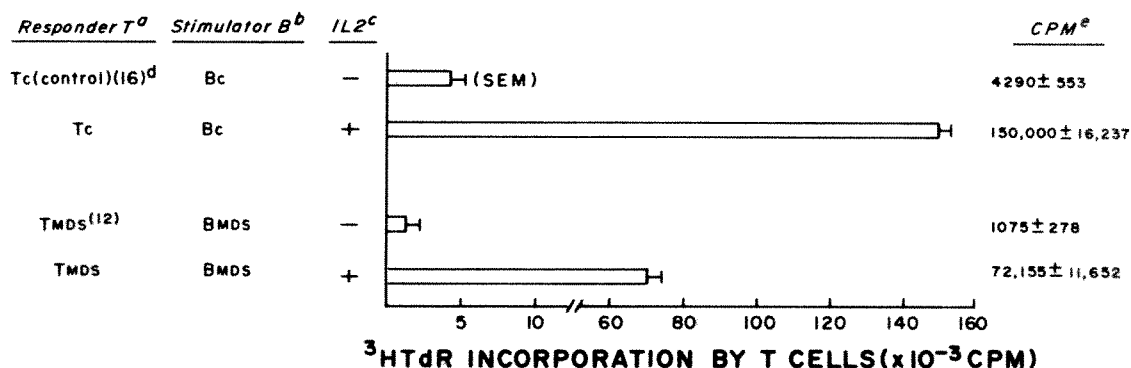


Fig 1. Response of Auto MLR to IL2 in MDS.

The presence of IL 2R on activated T cells was determined using a MoAb, anti-CD25, with specificity for the IL 2R.^{38,39} Table 4 shows the percentage of IL 2R expressing nonactivated, auto MLR-activated, or allo MLR-activated T cells from patients and compares them with identically activated control T cells. In these experiments, MDS or control T cells were stimulated with autologous or allogeneic B cells, and expression of IL 2R on activated T cells was determined by dual immunofluorescence. Results of these experiments are summarized in Table 4 and demonstrate that MDS T cells are unable to generate IL 2R when stimulated by autologous B cells, whereas they are able to develop normal IL 2R positivity when stimulated by control B cells. This was not due to the defective stimulatory activity of MDS B cells alone, since these B cells could activate normal T cells to develop IL 2R. Therefore, although MDS B cells are defective stimulators of IL 2 production by normal T cells, they are able to stimulate normal generation of IL 2R by these cells. In addition, we did not find a statistically significant difference in the proliferative rate of T cells and the stimulatory capacity of B cells between patients with refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) and those with refractory anemia with excess blasts (RAEB) in the auto or allo MLR cultures.

To verify these findings, we added recombinant human IL 2 to MLR cultures. The addition of various concentrations of recombinant IL 2 increased the T cell proliferation in both patient and control MLR cultures. In the auto MLR of MDS patients, although the T cell proliferation increased with the addition of IL 2, this proliferative rate was still significantly lower than that of the control cultures (Fig 1). In the allo MLR with MDS T cells as responders and normal B cells as stimulators, proliferation in experimental and control cultures was similar (Fig 2). These results confirmed the presence of impaired T and B cell interactions in MDS; ie, in the auto MLR, T cell IL 2R generation is impaired. Therefore, in these cultures, the low T cell proliferation cannot be completely reversed by exogenous IL 2. In contrast, when stimulated by control B cells, MDS T cells develop normal IL 2R positivity and proliferate in response to IL2.

The addition of IL 2 to allow MLR cultures containing normal T cells and MDS B cells corrected the low proliferative response to control levels (Fig 2), confirming the pres-

ence of adequate IL 2R on the T cells stimulated by MDS B cells.

DISCUSSION

This study demonstrates the presence of two major lymphocyte abnormalities in patients with MDS. The first abnormality is lymphopenia, involving B cells, T cells of both helper/inducer (CD4) and cytotoxic/suppressor (CD8) subsets, and NK cells. The second abnormality is an ineffective interaction between T and B cells, an interaction that is necessary for optimal immune responses.

Our results are in agreement with previous reports of lymphopenia.⁸⁻¹² The pathogenesis of the lymphopenia is unknown. In our patients, both CD4 and CD8 T cells were decreased in numbers to a similar extent, resulting in a normal ratio of CD4 to CD8 T cells. This is in contrast to studies in which T cells with a helper phenotype were diminished while those with a suppressor phenotype were normal or increased.^{8,9} Variations in phenotype may reflect the immunologic differences in each category of MDS. Alterations of lymphocyte subset distribution in the peripheral blood occur in the recipients of blood products,^{31,32} such that the degree of inversion in the ratio of CD4 to CD8 T cell ratio correlated with the number of RBC transfusions received by MDS patients.⁸ We did not find such a correlation in our study between transfusion and an inverted ratio of CD4 to CD8 T cells. Because many of our transfused patients had long-standing (>2 years) MDS, during this period myelodysplastic lymphopoiesis may have resulted in lymphopenia of both helper and suppressor T cell subsets, masking an initially present T cell subset abnormality.

Alterations in T and B interactions in MDS patients were studied with the use of the auto MLR. Abnormalities of this reaction have been reported in various immunologic and hematologic diseases,^{40,41} suggesting its possible role in vivo as a mechanism of immunoregulation. In patients with MDS, T cell responsiveness in auto MLR was significantly diminished as compared with that of age-matched controls. The low T cell proliferation in the auto MLR could not be due to a lack of CD4 T cells, which are thought to proliferate in this reaction, since their ratio to CD8 cells was unchanged. In addition to a low proliferative response, IL 2R generation by T cells in the auto MLR was also impaired,

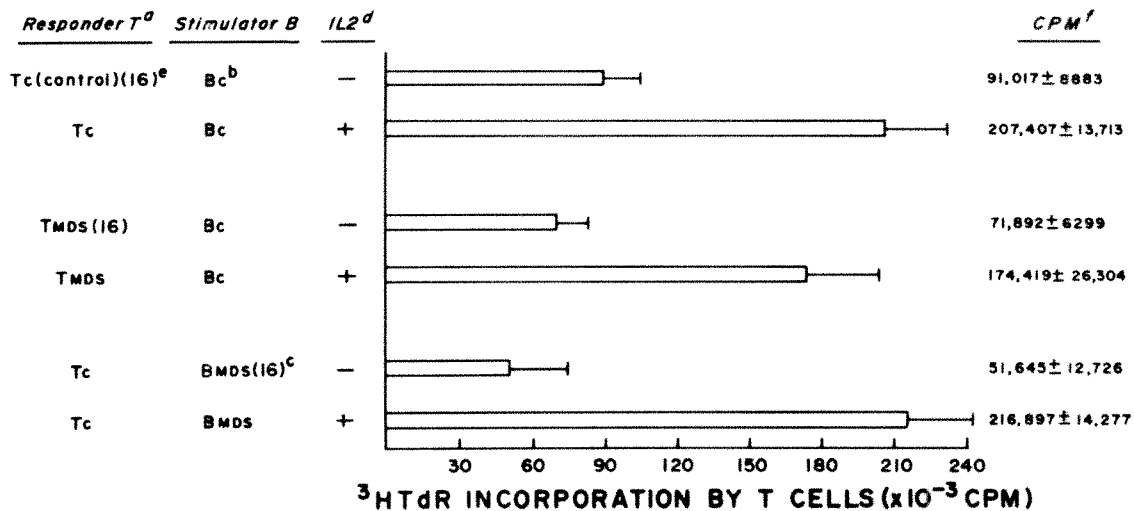


Fig 2. Response of Allo MLR to IL2 in MDS.

resulting in their failure to respond to exogenous IL 2 addition. Inability of MDS T cells to become activated in an auto MLR suggests that regulatory T cell generation in these patients is impaired. T cell responsiveness to self class II MHC antigens expressed by B cells and macrophages is particularly important because antigen is recognized by T cells in the context of their antigen receptors and the cell membrane DR determinants on the antigen-presenting cell.⁴⁰ The inability to respond to self class II MHC antigens could lead to impaired T cell responsiveness to foreign antigen. The result could be an inability to cope with viral, fungal, and other infective agents, as occurs in MDS patients.

In contrast to their poor responsiveness in the auto MLR, MDS T cells generated IL 2R, produced IL 2, and proliferated normally when stimulated by control B cells. These experiments showed that MDS T cells could be activated by foreign cellular antigens but not by autologous DR antigens and suggested that B cells from MDS patients were defective in their ability to activate MDS T cells.

To pursue a possible B cell defect, we examined the capacity of B cells from MDS patients to elicit a proliferative response by allogeneic T cells in addition to autologous T cells. B cells were significantly impaired in both capacities. The diminished proliferation was most likely due to the inability of MDS B cells to induce the generation of normal quantities of IL 2 from control T cells, since the expression of IL 2R by these cells was not defective. Thus, the addition of IL 2 normalized the T cell proliferation in these cultures.

Table 3. IL 2 Production by T Cells in MDS

Source of T Cells	T Cells Stimulated With		
	PHA/PMA (U/mL)	Control Allogeneic B Cells (U/mL)	MDS B Cells (U/mL)
C (5)	2.44 ± 0.4	1.87 ± 0.3	0.50 ± 0.1
MDS (5)	1.92 ± 0.7	1.42 ± 0.2	ND

Number of persons studied given in parentheses.

Abbreviations: PHA, phytohemagglutinin; PMA, phorbol myristate acetate.

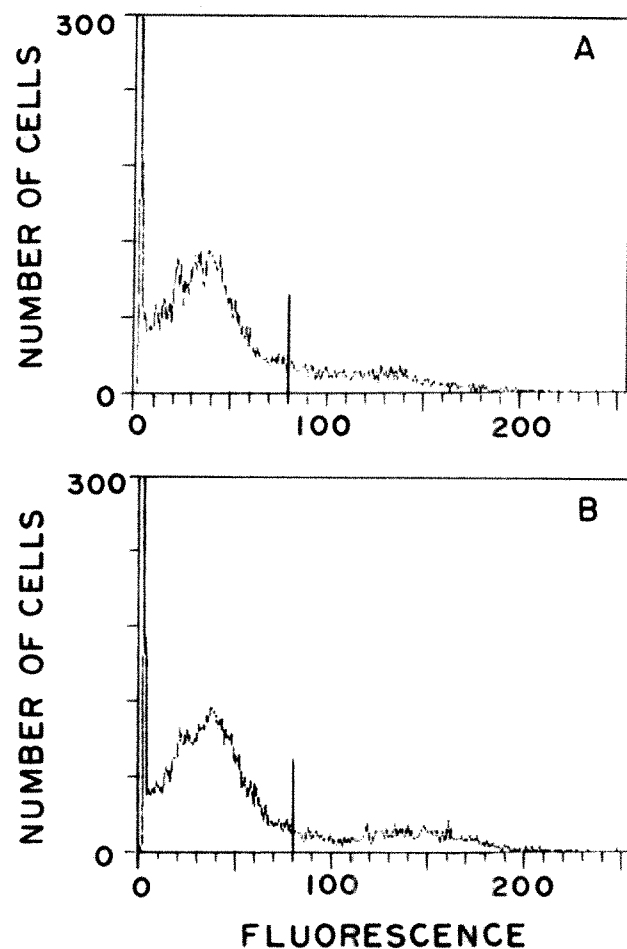


Fig 3.

Table 4. T Cell Activation in MDS Patients

Source of T Cells	IL 2R (CD25)-Positive T Cells (%) Activated With			
	Autologous B Cells*	Allogeneic B Cells†	MDS B Cells‡	Unactivated T Cells
C (5)	12 ± 2	22 ± 3	18 ± 2	<1.5%
MDS (5)	3 ± 2	20 ± 2	ND	<1.5%

Number of persons studied is given in parentheses. IL 2 receptor (IL 2R)-positive T cells were determined by dual immunofluorescence using a monoclonal anti-IL 2R antibody and anti-Leu 5.

*T cells (2×10^6 /mL) were stimulated with mitomycin C-treated autologous B cells (2×10^6 /mL) for 6 days.

†T cells (2×10^6 /mL) were stimulated with mitomycin C-treated allogeneic B cells (2×10^6 /mL) for 6 days.

‡Control T cells (2×10^6 /mL) were stimulated with mitomycin C-treated MDS B cells for 6 days.

Although all patients expressed normal concentrations of DR antigens on their B cells and macrophages, structural abnormalities not detectable by immunofluorescence in the DR determinants may exist or these MDS cells may lack a DR-linked determinant, such as DQ or DP, which could be required for stimulation of IL 2 production by T cells. Abnormalities of B cells, such as lack of surface EBV receptors in MDS patients, suggest that these cells are relatively immature. We previously showed that B cells in chronic lymphocytic leukemia (CLL) patients are also poor stimulators of IL 2 production but not IL 2R generation in normal T cells.⁴¹ CLL B cells are not fully mature and, as MDS B cells, also express DR antigens. The capacity of stimulation of IL 2 production by T cells may be acquired only by the fully mature B cells and therefore is lacking in MDS B cells and CLL cells, whereas stimulation of IL 2

expression is acquired earlier and is present in MDS and CLL B cells.

These results raise two important questions: (a) Why are lymphocytes deficient in number and impaired in function in MDS? (b) Do immunoregulatory abnormalities, ie, defective T and B cell interactions, affect the course of MDS? Lymphocytes in MDS patients may be quantitatively and functionally abnormal because they descend from an abnormal stem cell. Lack of self-recognition, as demonstrated by a low auto MLR and impaired regulatory T cell generation, exists in malignancies such as CLL, Hodgkin's disease, and colon carcinoma.⁴² The absence of regulatory T cells in these disorders may allow cells that carry phenotypic or karyotypic mutations to escape surveillance and other regulatory differentiation effects the T cells induce. In primary MDS, abnormal clones with karyotype aberrations occur in 50% of patients,^{3,4,43} and their presence is associated with leukemic change and a more rapid progression of the disease. Although the relationship between prognosis and cytogenetic abnormalities is established,^{3,4} the correlation of cytogenetic abnormalities with T cell regulatory functions and their relationship to the prognosis has not been studied. Surveillance by regulatory T cells may be necessary to prevent the unstable hemopoietic stem cells with karyotype abnormalities from gaining a growth advantage and developing leukemic transformation.

MDS offers a unique opportunity to study the changes in immunoregulatory T and B cell interactions in a preleukemic state. Investigating the mechanisms of effector and regulatory lymphocyte defects in these patients will contribute to our understanding of the contribution of these cells to the regulation of normal and leukemic hemopoiesis.

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Isolation, Characterization, and Localization of Glycosaminoglycans in Rabbit Bone Marrow

By Kayoko Oguri, Eiko Okayama, Bruce Caterson, and Minoru Okayama

The glycosaminoglycans that exist in rabbit bone marrow were analyzed chemically, and their in situ localization was studied immunohistochemically. Femoral bone marrow of 3-month-old rabbits was defatted with organic solvents. Glycosaminoglycans were prepared from the defatted tissue after its digestion with pronase, treatment with mild alkali, and then digestion with DNase-I. The tissue contained glycosaminoglycans equivalent to 195 mg of hexosamine per femur, which accounted for 27.3% of the total hexosamine in the tissue. Studies with hyaluronidase from *Streptomyces hyalurolyticus* and chondroitinase ABC showed that the glycosaminoglycans were composed of hyaluronic acid (16% of the total glycosaminoglycan) and chondroitin 6-sulfate (79%). The chondroitin 6-sulfate was separated on Bio-Gel A-0.5m gel into two molecular spe-

cies with mol wt of $>12,000$ ($K_d > 0.2$) and $\sim 8,000$ ($K_d = 0.47$). Bone marrow digested with chondroitinase ABC and then treated with three monoclonal antibodies 4/8/9-A-2, 5/6/3-B-3, and 5/6/1-B-5, which were specific for unsaturated 4-sulfated, 6-sulfated, and nonsulfated disaccharide structures, respectively, at the nonreducing end of chondroitin sulfate chains, reacted with only 5/6/3-B-3. This result indicated that the chondroitin sulfate isomer in the bone marrow is chondroitin 6-sulfate, consistent with the biochemical results. The chondroitin 6-sulfate was localized mainly in the extracellular compartment and was considered to be involved in construction of the hemopoietic microenvironment in the bone marrow.

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EVIDENCE shows that proliferation, differentiation, and maturation of cells are intimately regulated by the extracellular matrix that forms their microenvironment.^{1,2} In hemopoietic organs such as the bone marrow and spleen, the extracellular matrix has been proposed to be important in regulation of hemopoiesis as a hemopoietic inductive microenvironment³ and is considered to be formed by stromal cells.⁴ The idea of the crucial role of this microenvironment in hemopoiesis is supported by the finding that in long-term bone marrow cultures the maintenance of proliferation and differentiation of hemopoietic stem cells depends strictly on the presence of marrow-derived adherent (stromal) cells.⁵⁻⁸ In such cultures, the cloned adherent cells, including adipocytes, reticular cells, fibroblasts, and endothelial cells produce extracellular matrix components such as collagens, laminin, and fibronectin and deposit them as extracellular matrices.⁹

Using bone marrow from mice with a genetic defect (W/W^v) in production of hemopoietic cells, or from normal mice cultured in conditions in which no hemopoiesis occurred, Gallagher and colleagues¹⁰ demonstrated that glycosaminoglycans are also produced by the stromal cells themselves. In histochemical studies, McCuskey and colleagues demonstrated the presence of glycosaminoglycans in murine spleen and bone marrow in situ and specific alterations in their amount when erythropoiesis was regenerated, suppressed, or stimulated.^{11,12} Ploemacher and associates¹³ demonstrated that in vitro colony formation by erythroid and granuloid cells was affected by exogenous addition of various kinds of glycosaminoglycans. In particular, they observed the requirement of threshold concentrations of chondroitin sulfate isomers for stimulation and suppression of colony formations by bone marrow cells in culture. Spooncer and co-workers¹⁴ reported that the increased synthesis of chondroitin sulfate by adherent cells in long-term cultures supplemented with β -xyloside, which is an artificial initiator of chondroitin sulfate synthesis^{15,16} and causes suppression of production of proteoglycans by the cells, is associated with increases in the numbers of pluripotent stem cells (CFU-S), granulocyte progenitor cells (GM-CFC), and their mature progeny. This stimulation of hemopoiesis by

β -xyloside was dose dependent, and the optimal concentration (5×10^{-4} mol/L) for stimulation coincided with that for elongation of chondroitin sulfate chains.

As yet, however, the glycosaminoglycans present in hemopoietic organs have been poorly characterized quantitatively and qualitatively. Therefore, in this study, we examined the nature, quantities, and distributions of glycosaminoglycans in rabbit femoral bone marrow. Results showed that chondroitin 6-sulfate and hyaluronic acid were the predominant glycosaminoglycans in the tissue. The chondroitin 6-sulfate was found only in the extracellular matrix of the bone marrow.

MATERIALS AND METHODS

Materials. Three-month-old female albino rabbits (1.8 kg body weight) were obtained from a local animal supplier. Chondroitinase ABC, chondroitinase AC-II, chondro-6-sulfatase, chondro-4-sulfatase, hyaluronidase from *Streptomyces hyalurolyticus*, and the standard unsaturated disaccharides, 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyl)-6-O-sulfo-D-galactose (Δ Di-6S), 2-acetamido-2-deoxy-3-O-(β -D-glucopyranosyl)-

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ronic acid)-4-*O*-sulfo-D-galactose (Δ Di-4S) and 2-acetamido-2-deoxy-3-*O*-(β -D-gluc-4-enopyranosyluronic acid)-D-galactose (Δ Di-OS) for paper chromatography were purchased from Seikagaku Kogyo (Tokyo). Heparins of known mol wt (HA-I, HA-II, HA-III, LA-IV, and LA-V, with mol wt 22,000, 13,000, 9,400, 7,300 and 5,700, respectively) were generous gifts from Dr Suguru Suzuki of Eizai Co. (Tsukuba). Pronase was obtained from Kaken Kagaku (Tokyo), and DNase-I was obtained from Takara Biochemicals (Kyoto). Horseradish peroxidase-conjugated rabbit anti-immunoglobulin to mouse immunoglobulin was purchased from Dakopatts a/s (Copenhagen). Monoclonal antibodies 4/8/9-A-2, 5/6/3-B-3, and 5/6/1-B-5 (designated as 9-A-2, 3-B-3, and 1-B-5, respectively, in this article) to unsulfated 4-sulfated, 6-sulfated and nonsulfated chondroitin sulfate, respectively, were prepared and their epitopes were identified as described previously.^{17,19}

Preparation of samples for analyses of various components in bone marrow tissue. For preparation of samples for analyses of various components in bone marrow tissue, nine femora were processed separately as described in Fig 1. Both ends of the femora were cut off, and the bone marrow was washed out with phosphate-buffered saline (PBS). The bones were then washed and dried with acetone, and their length and the volume of their marrow cavity were measured. The marrow tissue was defatted by successive treatments twice each at -20°C with acetone, ether, chloroform/methanol (2:1, vol/vol) and acetone. The acetone powder was dried at room temperature and weighed. The defatted tissue was processed as described in Fig 1 and assayed for individual components as described below.

Enzymatic digestions. Pronase digestion was carried out with the enzyme ($1/25$ of dry weight or $1/50$ of protein), which was preincu-

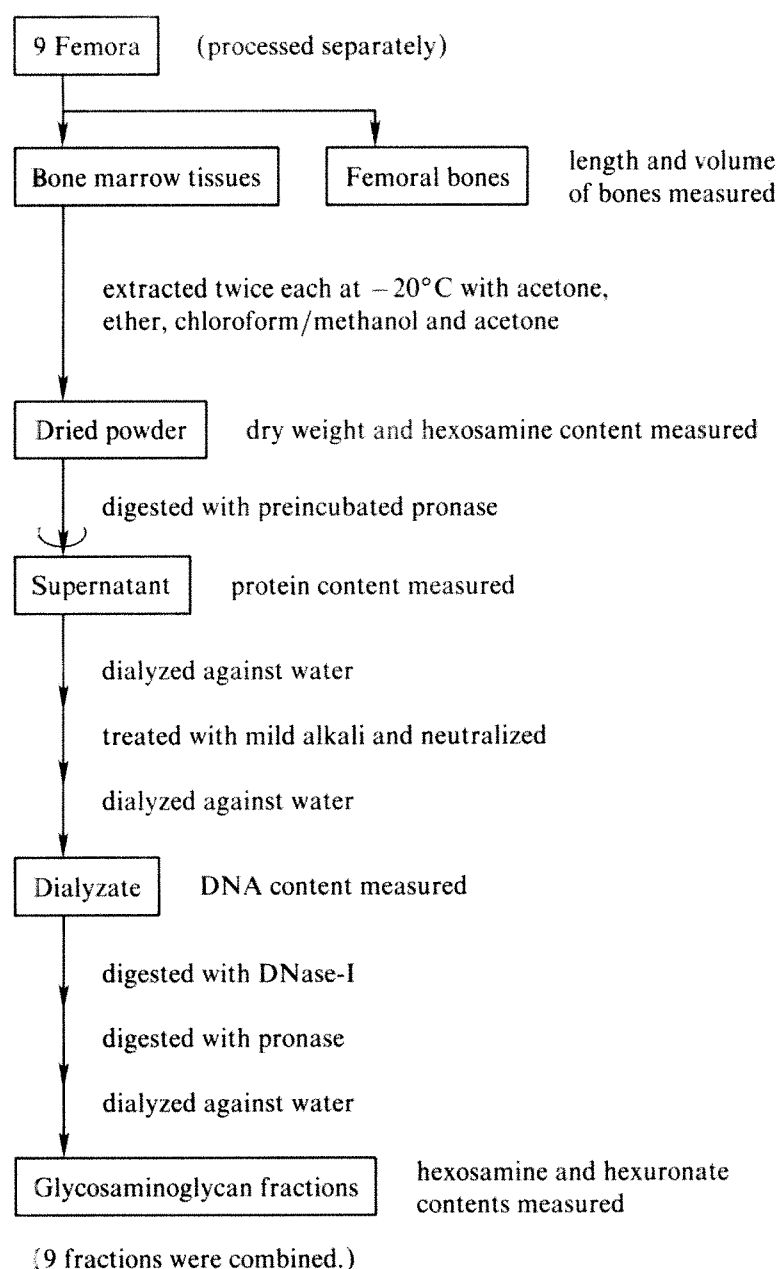


Fig 1. Isolation of glycosaminoglycans from rabbit bone marrow.

bated for 1 hour at 50°C to eliminate the activity of contaminating DNase, in 2 mmol/L of CaCl₂ and 0.1 mol/L of Tris-HCl, pH 8.0, at 50°C for 70 hours with two further additions of the same amount of the enzyme at 24-hour intervals. Digestions with chondroitinase ABC and chondro-6-sulfatase were carried out as described previously.²⁰ Hyaluronidase digestion was performed as described elsewhere.²¹ Digestion with DNase-I (200 U/mg of DNA equivalent to dAMP) was carried out in 5 mmol/L of NaCl and 0.1 mol/L of sodium acetate, pH 5.4, at 37°C for 1 hour.

Analytical methods. Protein was measured by the method of Lowry and associates.²² Hexuronate was determined by the carbazole method of Bitter and Muir.²³ DNA was determined by the method of Hinegardner,²⁴ with dAMP as a standard. Hexosamine was determined by the Elson-Morgan method²⁵ as modified by Boas²⁶ with galactosamine as a standard, after hydrolysis of the samples. In brief, the samples were hydrolyzed with 3 mol/L of HCl in evacuated tubes at 100°C for 7 hours. The hydrolysates were adjusted to a concentration of 0.5 mol/L of HCl, and adsorbed to a column (0.7 × 15 cm) of AG 50W × 4 (200 to 400 mesh, H⁺ form). The column was washed thoroughly with water, and then hexosamine was eluted with 2 mol/L of HCl. An aliquot of the eluate with 2 mol/L of HCl was dried on NaOH and dissolved in water, and its hexosamine content was determined.

Histochemical and immunohistochemical methods. Small pieces of bone marrow were taken out and fixed in periodate-lysine-paraformaldehyde, pH 7.4, for 1 hour at 4°C.²⁷ After dehydration by passage through an ascending alcohol series, the specimens were embedded in paraffin and sectioned at 3 μm. The sections were deparaffinized in xylene, hydrated in a descending alcohol series, stained with alcian blue, or treated with monoclonal antibodies.

Alcian blue staining was carried out by the method of Lev and Spicer,²⁸ before and after digestion of the preparations with chondroitinase. The sections were incubated with or without chondroitinase ABC (1 U/mL of 0.1 mol/L of Tris-HCl, pH 7.4, containing proteinase inhibitors²⁹) at room temperature for 10 hours. They were then washed with water and stained with 1% (wt/vol) alcian blue 8GX in 0.1 mol/L of HCl for 30 minutes at room temperature. Preparations were counterstained with Kernechtrot.

The distributions of chondroitin sulfate isomers were examined with monoclonal antibodies 9-A-2, 3-B-3, and 1-B-5 as follows: The specimens were digested with chondroitinase ABC (0.05 U/mL of 0.1 mol/L of Tris-HCl, pH 7.3, containing proteinase inhibitors) for 5 minutes at room temperature, incubated with monoclonal antibodies for 1 hour, washed for 30 minutes in 0.15 mol/L of phosphate-buffered saline (PBS), and then incubated with a solution of horseradish peroxidase-conjugated second antibody. Sections were washed with 50 mmol/L of Tris-HCl, pH 7.4, and color was developed in 3,3'-diaminobenzidine solution containing 0.3% H₂O₂. Sections were counterstained with Giemsa. Control sections were processed in the same manner, except that they were treated with buffer without the enzyme.

Other methods. Two-dimensional electrophoresis of glycosaminoglycan samples was carried out on a cellulose acetate membrane as described previously.³⁰ Paper chromatography was carried out on Toyo No 51A paper in 1-butyric acid/acetic acid/1 mol/L of NH₄OH (2/3/1).

RESULTS

Glycosaminoglycan content of bone marrow. To prepare glycosaminoglycans from bone marrow and to determine their contents and those of various other compounds in bone marrow, we processed nine femora of 3-month-old rabbits separately, as shown in Fig 1. The length of femur cut at both

ends was 4.00 ± 0.11 cm, and the volume of the marrow cavity was 0.99 ± 0.10 cm³. Bone marrow tissue was defatted by extraction with organic solvents as described in the Materials and Methods section and dried. The dry weight of the defatted powder was 116.0 ± 20.0 mg/femur. Analysis of parts of defatted powder of individual samples showed that the total hexosamine content was 0.61 ± 0.73 mg/100 mg dry weight of tissue. The remaining portions of the samples were suspended in water, boiled for 5 minutes to denature proteins, adjusted to 0.1 mol/L of Tris-HCl, pH 8.0, and digested extensively with pronase. The preparations were then centrifuged to remove insoluble materials, and the protein contents of aliquots of the supernatants were measured. The values obtained were corrected by subtracting the protein content of the control containing enzyme only. The protein content was 18.0 ± 1.9 mg/100 mg dry weight of tissue. This value was almost the same as that determined using aliquots of the suspensions before centrifugation, indicating that pronase digestion for 70 hours was sufficient to solubilize all the tissue protein. After dialysis, the dialyzates were treated with mild alkali with 1 mol/L of NaBH₄ added to release glycosaminoglycans and to degrade RNA in the samples. The DNA content of these samples was determined as described by Hinegardner.²⁴ In this method, sialic acid from glycoproteins, which also shows fluorescence, was removed by the alkali treatment and dialysis step. The DNA content was 2.46 ± 0.44 mg equivalents of dAMP per 100 mg dry weight. DNA in the samples was digested with DNase-I; the enzyme was then degraded by pronase digestion, and resultant small molecules were removed by dialysis. The dialyzates were freeze-dried, dissolved in water, and used as glycosaminoglycan samples. The hexosamine content of these glycosaminoglycan samples was 0.168 ± 0.012 mg/100 mg dry weight of tissue. The hexosamine content of glycosaminoglycan fraction was ~27% of the total hexosamine. The remaining hexosamine (73%) was assumed to be derived from sugar moieties of glycoproteins in the defatted tissue. The hexuronate content of the glycosaminoglycan samples

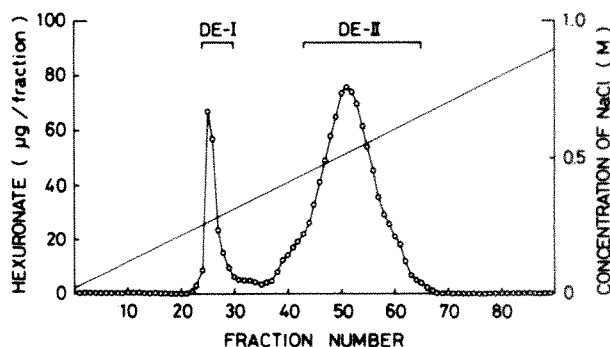


Fig 2. Diethylaminoethanol (DEAE)-Sephacel column chromatography of glycosaminoglycans isolated from rabbit bone marrow. The glycosaminoglycan fractions from nine femora (shown in Fig 1) were combined and chromatographed on a DEAE-Sephacel column (0.7 × 17 cm). Material was eluted at 5 mL/h with 200 mL of a linear gradient of 20 mmol/L to 1 mol/L of NaCl. Fractions of 2 mL were collected. The hexuronic acid content of the eluate was monitored. Fractions DE-I and DE-II (bars) were pooled for further study.

was 0.189 ± 0.017 mg/100 mg dry weight of tissue. The molar ratio of hexuronate to hexosamine in the samples was ~ 1 , indicating that the glycosaminoglycans in the samples were purified considerably. The concentration of hexuronate in the marrow cavity was 0.191 ± 0.017 mg/mL.

Isolation and characterization of glycosaminoglycans. The nine individual glycosaminoglycan samples were combined and subjected to ion-exchange chromatography on diethylaminoethanol (DEAE)-Sephacel (Fig 2). Hexuronate in the fractions was monitored. On elution with a linear concentration gradient of NaCl, materials containing hexuronate were eluted in two fractions: fraction DE-I eluted with 0.25 to 0.3 mol/L of NaCl as a sharp peak, and fraction DE-II eluted with 0.4 to 0.65 mol/L of NaCl as a rather broad peak. Recovery was $\sim 90\%$ as hexuronate. Aliquots of DE-I and DE-II were digested with hyaluronidase or chondroitinase ABC, respectively, and these digests were then chromatographed on Bio-Gel P-6 (Fig 3). Glycosaminoglycan in DE-I was completely degraded by hyaluronidase and was identified as hyaluronic acid (Fig 3A), and that in DE-II was depolymerized by chondroitinase ABC and identified as

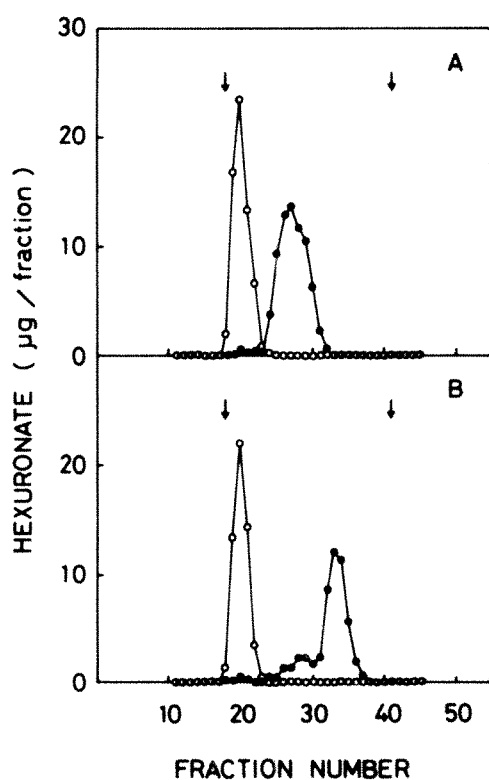


Fig 3. Bio-Gel P-6 gel chromatography of glycosaminoglycans treated with and without hyaluronidase or chondroitinase ABC. (A) Aliquots of the pooled fraction, DE-I, on diethylaminoethanol (DEAE)-Sephacel were treated with (●) and without (○) hyaluronidase and applied to a column (0.8×46 cm) of Bio-Gel P-6, which was equilibrated and eluted with 0.5 mol/L of NaCl in 0.1 mol/L of Tris-HCl, pH 7.3, at a flow rate of 2.3 ml/h. Fractions of 0.56 mL were collected. (B) Aliquots of the DE-II fraction, on DEAE-Sephacel, were treated with (●) and without (○) chondroitinase ABC and applied to the same column. Hexuronic acid in the eluate was monitored. Arrows show the void volume and total volume of the column.

chondroitin sulfate isomers (Fig 3B). Thus, the glycosaminoglycans in rabbit bone marrow contain 79% chondroitin sulfate and 16% hyaluronic acid (Table 1). The remaining 5%, which was eluted between DE-I and DE-II from DEAE-Sephacel (Fig 2), was not studied further.

The chondroitin sulfates of the DE-II fraction were characterized further. After gel filtration on a Bio-Gel A-0.5 m column previously calibrated with heparins of known mol wt, the chondroitin sulfates were separated into two fractions (Fig 4A), fraction DE-II-A (18% of the applied hexuronate) and fraction DE-II-B (81%), respectively. The apparent mol wt of the former peak was estimated as $>12,000$, and that of the latter was estimated as $\sim 8,000$ from the calibration curve (inset in Fig 4A). On two-dimensional electrophoresis, the materials in DE-II-A migrated as one major spot with three other tiny spots (Fig 4B). All these spots disappeared after digestion of the sample with chondroitinase ABC (data not shown), indicating that the chondroitin sulfates in this fraction are heterogeneous in both chain size and charge density. The chondroitin sulfate in DE-II-B migrated as a single spot with a tail (Fig 4C). This spot also disappeared after digestion of the sample with chondroitinase ABC. These results indicate that the chondroitin sulfates in bone marrow consist of several molecular species differing in molecular size and charge density. To demonstrate the isomeric structures of these chondroitin sulfates in terms of the position of the sulfate residue, the samples were digested with chondroitinase ABC, chondroitinase AC-II, chondroitinase ABC plus chondro-6-sulfatase or chondroitinase ABC plus chondro-4-sulfatase, and the products were subjected to paper chromatography. As shown in Fig 5, after digestion with chondroitinase ABC, the chondroitin sulfates of the two fractions gave only one spot comigrating with the external reference Δ Di-6S, with no significant amounts of Δ Di-4S or Δ Di-OS (Fig 5, lanes a and c). The digests with chondroitinase ABC plus chondro-6-sulfatase comigrated with Δ Di-OS (Fig 5, lanes b and d). Digests with chondroitinase ABC plus chondro-4-sulfatase comigrated with Δ Di-6S, indicating that 4-sulfated disaccharides were not present (data not shown). These results indicated that all the chondroitin sulfates of the two fractions were 6-sulfated, and that the heterogeneity observed on two-dimensional electrophoresis was due solely to differences in their chain sizes. Similar results were obtained on digestion of the samples with chondroitinase AC-II, chondroitinase AC-II plus chondro-6-sulfatase, and chondroitinase AC-II plus chondro-4-sulfatase (data not shown). These results indicate that the bone marrow contained little, if any, dermatan sulfate. We demonstrated that the chondroitin 6-sulfates in bone marrow

Table 1. Molecular Species of Glycosaminoglycans Contained in Rabbit Bone Marrow Tissue

Molecular Species	Hexuronate/Femur (µg)	Ratio (%)
Chondroitin sulfate	173.4	79.1
Hyaluronic acid	34.8	15.9
Unidentified glycosaminoglycans	11.0	5.0

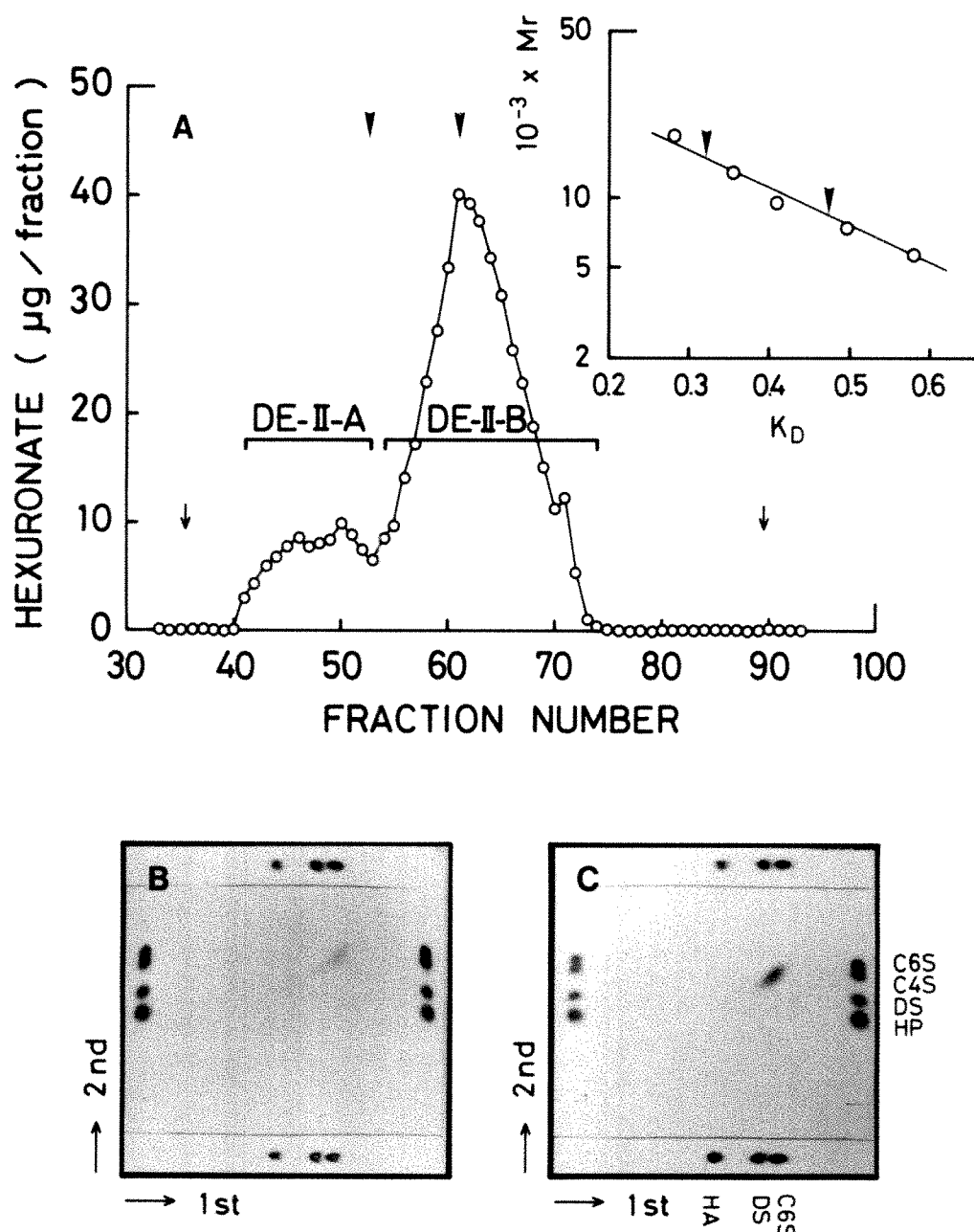


Fig 4. Bio-Gel A-0.5m gel chromatography and two-dimensional electrophoresis of the glycosaminoglycans of the DE-II pooled fraction from diethylaminoethanol (DEAE)-Sephacel. Fraction DE-II from DEAE-Sephacel was adjusted to 1 mol/L of NaCl in 50 mmol/L of Tris-HCl, pH 7.3, and chromatographed on a column (1 $\times$ 115 cm) of Bio-Gel A-0.5m (A), which was equilibrated and eluted with 1 mol/L of NaCl in 50 mmol/L Tris-HCl, pH 7.3, at a flow rate of 3.9 mL/h. Fractions of 1.4 mL were collected. Hexuronic acid in the eluate was monitored. Arrows show the void volume and total volume of the column. Fractions DE-II-A and DE-II-B, indicated with bars, were pooled for further study. The column was previously calibrated with heparins of known mol wt (22,000, 13,000, 9,400, 7,300, and 5,700). The inset in panel A shows the calibration curve obtained by plotting K_D (abscissa) v mol wt (on a logarithmic scale). Arrowheads indicate the K_D values of the two fractions DE-II-A and DE-II-B, respectively. Aliquots of DE-II-A (B) and DE-II-B (C) were subjected to two-dimensional electrophoresis on cellulose acetate membranes. The standard glycosaminoglycans used as external references were hyaluronic acid (HA), dermatan sulfate (DS), chondroitin 4-sulfate (C4S), chondroitin 6-sulfate (C6S), and heparin (HP).

consist of three molecular species of proteoglycans with different molecular wt.³¹

Distributions of glycosaminoglycans. The distributions of sulfated glycosaminoglycans in the bone marrow were first surveyed by alcian blue staining at pH 1.0, coupled

with chondroitinase digestion. Under these conditions, strongly acidified glycosaminoglycans such as sulfated ones are stained with the dye, but weakly acidified glycosaminoglycans or glycoproteins such as hyaluronic acid and sialoglycoproteins are not stained.²⁹ When specimens were

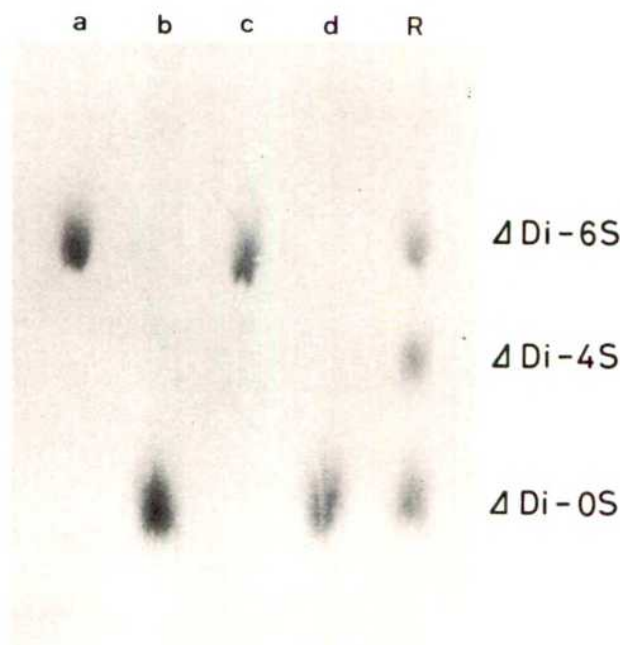


Fig 5. Paper chromatography of DE-II-A and DE-II-B glycosaminoglycan samples digested with chondroitinase ABC or chondroitinase ABC plus chondro-6-sulfatase. Aliquots of DE-II-A and DE-II-B were digested with the enzymes, and the products were chromatographed on Toyo No 51A paper. Lanes a and b are the digests of DE-II-A with chondroitinase ABC and chondroitinase ABC plus chondro-6-sulfatase, respectively, and lanes c and d are the digests of DE-II-B with chondroitinase ABC and chondroitinase ABC plus chondro-6-sulfatase, respectively. Lane R is a mixture of the unsaturated disaccharides Δ Di-OS, Δ Di-4S and Δ Di-6S, as external references.

stained with alcian blue, the cytoplasm of megakaryocytes and granuloid cells stained strongly and the extracellular compartment stained moderately (Fig 6A). After extensive digestion of specimens with chondroitinase ABC, no stained material was apparent in megakaryocytes or the matrix, but that in granuloid cells remained, indicating that the former were chondroitin sulfate isomers, but the latter was not (Fig 6B). For these studies, rabbit tracheal cartilage was used as a positive control. Alcian blue strongly stained the cartilage matrix under these conditions, and almost all the stained materials disappeared on digestion with chondroitinase (data not shown). The stained material in granuloid cells was not studied further.

Monoclonal antibodies specific for the chondroitin sulfate isomers were used to determine whether the chondroitin sulfate isomers shown histochemically to be present in the cytoplasm of megakaryocytes and in the extracellular matrix were chondroitin 6-sulfate. The three monoclonal antibodies used were 3-B-3, 9-A-2 and 1-B-5, which are specific for unsaturated 6-sulfated, 4-sulfated, and nonsulfated disaccharide structures, respectively.^{18,19} These structures appear at the nonreducing end of chondroitin sulfate chains after digestion with chondroitinase ABC. As shown in Figs 8B, D, and F, specimens that had not been digested with chondroitinase ABC did not react with any of these antibodies. After digestion with chondroitinase ABC, specimens reacted with

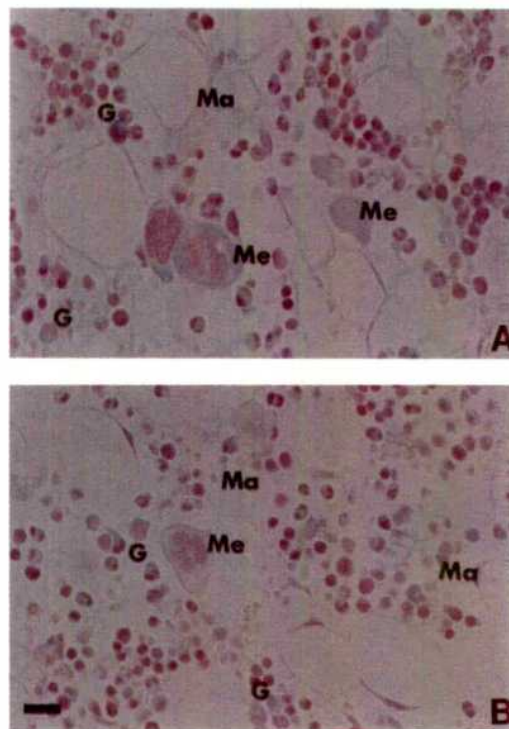


Fig 6. Alcian blue staining of the bone marrow before and after digestion with chondroitinase ABC. The bone marrow specimens were stained with alcian blue at pH 1.0 before (A) and after (B) digestion with chondroitinase ABC. Counterstaining was performed with Kernechtrot. Bar = 20 μ m. Me, megakaryocytes; G, granuloid cell; Ma, matrix.

3-B-3 (Fig 8A), but not with the other two antibodies (Figs 8C and E). Thus, the chondroitin sulfate isomer present on proteoglycans in the bone marrow is chondroitin 6-sulfate. However, because chains of chondroitin sulfates from other tissues consist of hybrid structures containing 6-sulfated, 4-sulfated, and nonsulfated disaccharides, the reactivities of the three antibodies were examined after the specimens had been digested with chondroitinase ABC to different degrees. The reactivities of these specimens with the three antibodies did not significantly change, using specimens treated with

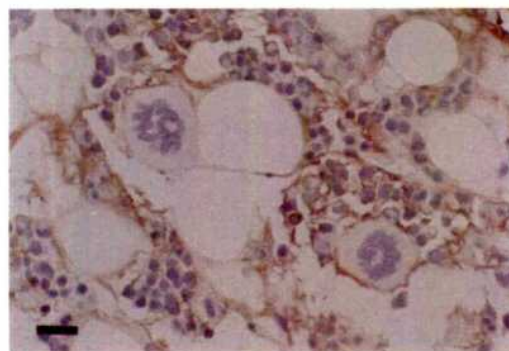


Fig 7. Higher magnification of immunochemical staining of the bone marrow with monoclonal antibody 3-B-3, after digestion with chondroitinase ABC. The bone marrow specimen was treated as described in Fig 6. Bar = 20 μ m.

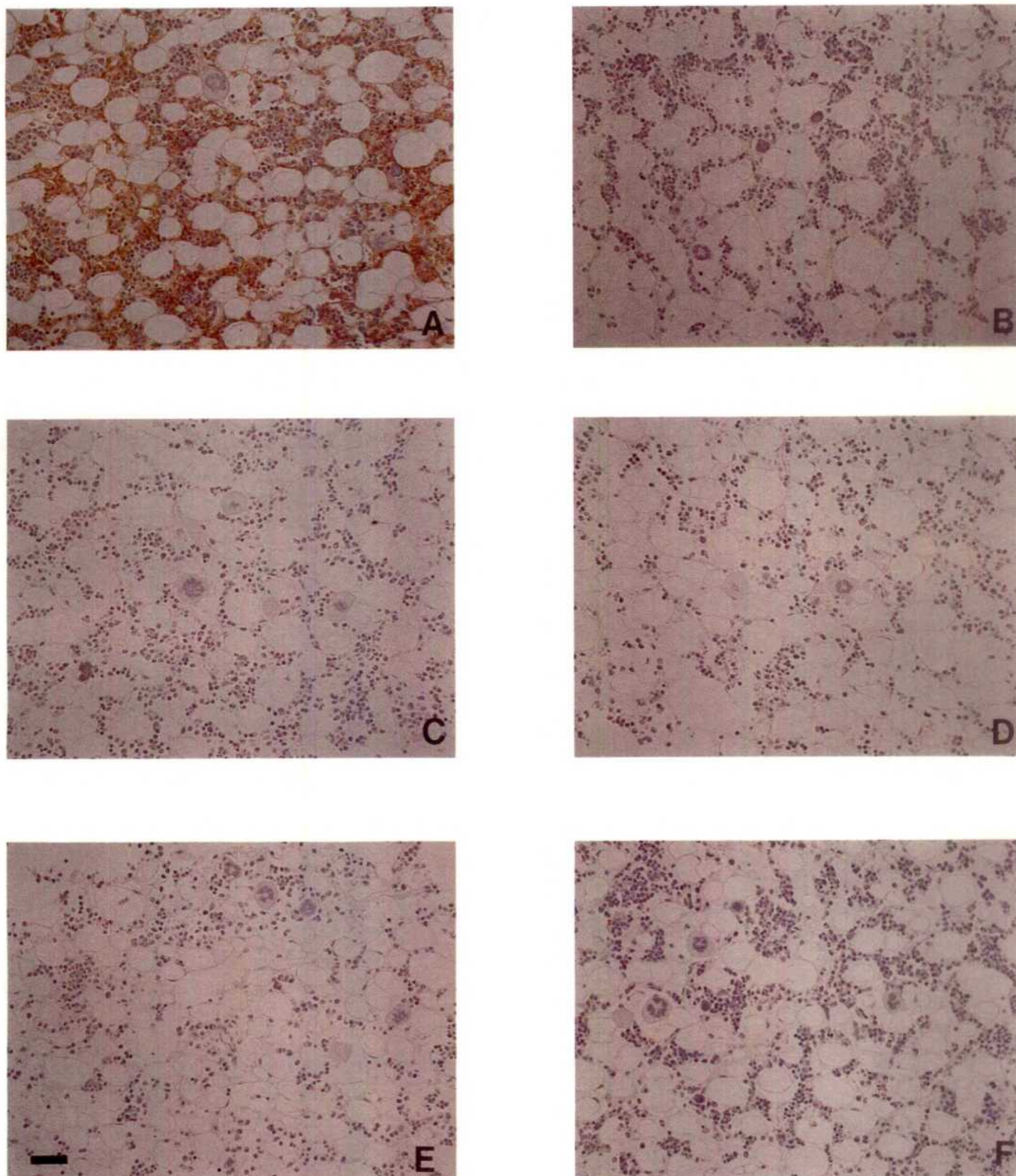


Fig 8. Immunochemical staining of bone marrow with monoclonal antibodies 3-B-3, 9-A-2, and 1-B-5, specific for unsaturated 6-sulfated, 4-sulfated, and nonsulfated disaccharide, respectively. Bone marrow specimens were treated with monoclonal antibody 3-B-3, 9-A-2, or 1-B-5, after treatment with (A, C, and E, respectively) and without (B, D, and F, respectively) chondroitinase ABC, and then treated with horseradish peroxidase-conjugated second antibody. Color was developed with diaminobenzidine solution containing H_2O_2 . Counterstaining was performed with Giemsa. Bar = 50 μm .

concentrations of chondroitinase ABC of 0.05 to 1 U/ml for 5 minutes, or for incubation times of 5 minutes to 10 hours with 0.05 and 1 U of the enzyme. This result indicates that the chondroitin sulfate chains of this tissue have unsaturated 6-sulfated disaccharides at their nonreducing end regardless of the different degrees of degradation of the chains. This is consistent with the biochemical result that the chondroitin sulfate isomer present in the bone marrow is fully sulfated chondroitin 6-sulfate and generates only unsaturated 6-sulfated disaccharides after digestion with chondroitinase ABC (Fig 5).

Higher magnification (Fig 7) showed that the materials reacting with 3-B-3 were localized in the matrix of the bone marrow and were somewhat concentrated in the periphery of certain hemopoietic cells. Although the cytoplasm of megakaryocytes contained chondroitinase-sensitive alcian blue-stainable materials (Fig 6A), megakaryocytes of chondroitinase-treated specimens did not react with any of the antibodies tested. The specimens were pretreated with several proteinases such as trypsin and pronase before chondroitinase digestion, because the chondroitinase-sensitive material in megakaryocytes was expected to be packed in granules with proteins that might prevent the interaction of antibodies with antigens. However, the three antibodies did not react with megakaryocytes under the conditions examined (data not shown).

Antibody 1-B-5, with specificity directed against the unsaturated nonsulfated disaccharide structure of chondroitin sulfate chains, cross-reacts with the unsaturated disaccharide units (unsaturated glucuronosyl *N*-acetylglucosamine) at the nonreducing end of hyaluronic acid chains, which appear after digestion with chondroitinase or bacterial hyaluronidase.¹⁹ Hyaluronic acid, which accounted for ~16% of the total glycosaminoglycan in the bone marrow, as described above, should have been detectable using this monoclonal antibody. This antibody, however, did not react with specimens pretreated with various concentrations of chondroitinase ABC. At present, whether hyaluronic acid is covalently linked to core protein has not been determined, but hyaluronic acid produced by fibrosarcoma appears to occur as free chains.^{32,33} If this is also the case in bone marrow, hyaluronic acid oligosaccharides with the epitope produced by digestion with chondroitinase ABC probably were washed out from the specimens. In the case of chondroitin 6-sulfate, the epitope produced by digestion with chondroitinase ABC would have been retained in the specimens in molecular forms of oligosaccharide-core protein complexes.³⁴

DISCUSSION

In this study, we found that the bone marrow of 3-month-old rabbits contained glycosaminoglycans equivalent to hexuronate 0.189 mg/100 mg dry weight of defatted tissue. The glycosaminoglycan was composed mainly of chondroitin 6-sulfate, accounting for 79% (equivalent to 0.15 mg of hexuronate), and hyaluronic acid, accounting for 16% (0.03 mg hexuronate). The hexosamine of these glycosaminoglycans accounted for ~27% of the total hexosamine

present in the defatted tissue. The remaining 73% of the hexosamine might be that of sugar moieties of glycoproteins in the defatted tissue. These values were consistent with those reported previously by other researchers for isolated rabbit bone marrow cells.^{35,36} Olsson³⁵ reported that rabbit bone marrow cells, obtained by filtration through silk cloth of a bone marrow suspension in Krebs-Ringer's-phosphate solution without calcium or magnesium, contained chondroitin 6-sulfate equivalent to 0.9 mg of hexuronate/g of fat-free cells and a hyaluronic acid-like glycosaminoglycan equivalent to 0.3 mg of hexuronate. Taniguchi and colleagues³⁶ estimated that rabbit bone marrow cells prepared by a method similar to that of Olsson contained glycosaminoglycans, mainly chondroitin 6-sulfate, equivalent to 1.5 mg hexuronate/g of fat-free cells. Olsson reported that >90% of the cells in her preparation were granuloid cells and that the glycosaminoglycans were tightly associated with these cells after dissociation into single cell suspension. The defatted samples obtained in the present study from the whole bone marrow were prepared in a similar way, and our values based on dry weight are comparable. A difference in the chondroitin 6-sulfate contents of whole bone marrow (1.5 mg/g of dry weight) and single cells (0.9 mg/g of dry weight) from marrow tissue might indicate that the chondroitin 6-sulfate is present in different forms in bone marrow. For instance, they could occur in a cell-associated form (60%) and an extracellular form (40%) that would be easily released on dissociation of the tissue into single cells. The amount of hyaluronic acid (0.3 mg/g dry weight) in the bone marrow was exactly the same as that of dissociated bone marrow cells reported by Olsson.³⁵ Thus, although we could not determine the localization of the hyaluronic acid in the tissue, it is probably associated with cells in the marrow tissue.

Only one of the monoclonal antibodies demonstrated localization of the chondroitin 6-sulfate. The chondroitin 6-sulfates were present entirely in the extracellular compartment of the bone marrow, indicating that they are involved in construction of the hemopoietic microenvironment. Previous studies in this laboratory and elsewhere demonstrated that the glycosaminoglycan side chains of proteoglycans contained in and/or produced by peripheral blood constituents, ie, mature blood cells, and contained in the plasma, are all chondroitin 4-sulfates or low-sulfated chondroitin 4-sulfates with little, if any, chondroitin 6-sulfate.³⁷⁻⁵¹ These results suggest that the chondroitin 6-sulfates present in bone marrow are not brought out into the peripheral blood by hemopoietic progeny cells and are not released into the plasma, but are involved in construction of the matrix of the bone marrow and may have an action only on cells in their immediate vicinity.

The chondroitin sulfate chains described in this study are constituted purely of 6-sulfated repeating disaccharide and of no detectable amounts of 4-sulfated and nonsulfated disaccharides. This kind of chondroitin sulfate isomer has not been reported in adult tissues. Sampaio and Dietrich⁵² reported that several tissues of bovine fetus contained the chondroitin sulfate isomer constituted of only 6-sulfated repeating disaccharide and the concentration of that

increased up to the 50th day of bovine fetal development and then decreased progressively until its complete disappearance in those tissues. They proposed that the chondroitin 6-sulfate acts as a stimulant of cell division. Hart⁵⁰ demonstrated that the major sulfated glycosaminoglycans synthesized by thymic lymphocytes were chondroitin 4-sulfate, but that the relative proportion of chondroitin 6-sulfate increased

dramatically when the cells were stimulated with mitogens. Together with these data, the present finding that chondroitin 6-sulfate is present in bone marrow, a tissue that unlike most other tissues in adults maintains continuous cell proliferation, suggests that this compound plays an important role in regulation of proliferation of hemopoietic cells.

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ADP Causes Subsecond Changes in Protein Phosphorylation of Platelets

By D.J. Carty, D.L. Freas, and A.R.L. Gear

We developed a general quenched-flow approach to study platelet function as early as 0.3 seconds after stimulation. Phosphorylation of 20- and 47-kiloDalton (kD) proteins was analyzed during the first 5 seconds of platelet response to ADP from 0.5 to 10.0 $\mu\text{mol/L}$ and compared with the progress of aggregation. The onset time for aggregation and phosphorylation of both proteins was <1 second; 20-K phosphorylation was increased $>200\%$ and 47-K phosphorylation was increased 50%. The ADP sensitivity of 20-K phosphorylation was greater than that of 47-K phosphorylation ($P < .025$), and of that of aggregation ($P < .01$), with

K_m values of 0.7, 1.0, and 1.2 $\mu\text{mol/L}$ of ADP, respectively. The cyclooxygenase inhibitor indomethacin had no effect on aggregation, but inhibited both phosphorylations. Its inhibition of 20-K phosphorylation was greater than that of 47-K phosphorylation. Platelet activation by ADP thus induced biochemical changes well before 1 second. The quenched-flow approach may help to reveal relationships between phospholipase activation, calcium fluxes, and protein phosphorylation during these early periods of platelet activation.

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ONE EARLY EVENT in blood platelet activation is the phosphorylation of certain platelet proteins, most prominently the 20-kiloDalton (kD) myosin light chain (MLC) and a 47-kD protein whose function remains uncertain,¹ but may be linked to secretion. Relationships between calcium fluxes and protein kinase activations are also of special interest.

To understand the interrelationships between these protein phosphorylations and other early events in platelet function, we developed a general quenched-flow approach² that allows analysis of platelet function^{2,4} and biochemical changes⁵ during the first seconds of response to stimulation. This time-scale approaches that suggested from theoretical calculations of the speed required for efficient hemostasis.^{6,7} We found that major morphological changes are nearly complete within 2 seconds after platelet activation by ADP or thrombin,² and that aggregation as detected by single-particle counting is $\sim 80\%$ complete within 5 seconds.^{2,4}

The quenched-flow system has been used to study the thrombin-induced phosphorylation of the 20- and 47-kD proteins.⁵ The onset time for both phosphorylations and aggregation is <1 second, and the maximum increase in phosphorylation during the first 5 seconds is similar for the two proteins. With <0.5 U/mL thrombin, however, 47-K phosphorylation lags behind 20-K phosphorylation, and significant phosphorylation occurs in the absence of significant aggregation. The thrombin sensitivity of both 20-K phosphorylation and aggregation is approximately twice that of 47-K phosphorylation.⁵ Because substantial differences appear to exist between the mechanisms of thrombin- and ADP-induced platelet activation,^{8,9} we examined early protein phosphorylations in response to ADP. Some of these data were presented in preliminary form.¹⁰

MATERIALS AND METHODS

Platelet preparation and labeling. Venous forearm blood (90 mL) was drawn from normal volunteers, anticoagulated with acid-citrate-dextrose (ACD), and platelet-rich plasma (PRP) was prepared as described previously.¹¹ Apyrase 7.5 U/mL (Sigma, Type VII, St Louis) and 0.3 mg/mL PGI_2 (Sigma) were added to remove released ADP¹² and to raise platelet cyclic AMP levels.¹³

The platelets were labeled by incubating 20 to 30 mL of PRP at 37°C for 60 minutes with 10 to 20 mCi [^{32}P]-inorganic phosphate (New England Nuclear, Boston). Extra ACD was added (1:10 final vol) to improve the percentage of platelets recovered and their final aggregability. The lowering of pH by ACD prevents premature

activation during subsequent centrifugations. The PRP was then centrifuged for 15 minutes at 350 g. Subsequently, the platelets were resuspended in ACD containing 7.5 U/mL apyrase, centrifuged again, and finally resuspended in an Eagle's salt solution (GIBCO, Grand Island, NY), buffered with HEPES (pH 7.2) containing human fibrinogen at 0.50 mg/mL¹⁴ and 0.05 U/mL hirudin (grade IV, Sigma). The labeled platelet-rich-Eagle's solution (PRE) was gassed with a 5% CO_2 and 95% air mixture to preserve platelet function,¹⁵ and aliquots of PRE (statics) were set aside to provide values for platelets not subject to quenched-flow. This procedure provides platelets that respond well to challenge by ADP and are not refractory.

Quenched-flow methods. Prior to any quenched-flow run, the PRE was incubated for 5 minutes at 37°C, sometimes with 1 $\mu\text{g/mL}$ indomethacin (Sigma). A concentration of 1 $\mu\text{g/mL}$ is sufficient for maximal inhibition of platelet cyclooxygenase.¹⁶ A 5-mg/mL solution of indomethacin in absolute ethanol was diluted to 1 mg/mL with saline and added to PRE at 1 $\mu\text{L/mL}$. The PRE was placed in one syringe of the quenched-flow apparatus described previously.^{2,4} The principle behind this system is to pump platelets through narrow-bore tubing (0.2 to 0.3 mm ID) together with inducing agent and to quench the reaction at the outlet. The shear stresses (34 dynes/cm² for 0.25 mm ID) in the reaction tubing are completely sufficient to ensure efficient mixing (<1 second⁵) and subsequent aggregation. The hydrodynamic conditions and basic results on aggregation kinetics have been fully reported.² A glutaraldehyde solution (3% vol/vol in 0.15 mol/L of NaCl) was used as quencher for assessing aggregation efficiency.⁴ Perchloric acid (3N in 0.15 mol/L of NaCl) was used to quench the reacting platelets for subsequent protein electrophoresis procedures. The speed of quenching in our system is ~ 0.1 second.⁵

Platelet aggregation. To evaluate the extent of platelet aggregation, 60 μL of the effluent from the quenched-flow apparatus were diluted to 10 mL with saline and analyzed in a resistive particle counter (Particle Data, Elmhurst, IL). Aggregation is expressed as the loss of singlet platelets at various reaction times, with the preaggregation value serving as 100%.² In these experiments, the

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inducing agent was either saline (as a control), or 0.5, 2, or 10 $\mu\text{mol/L}$ ADP in saline.

Protein phosphorylation. Protein phosphorylation was assessed as described previously.^{5,17}

Statistical analysis. Autoradiographic data were stored and manipulated by a Lotus 1,2,3 program. Student's *t* test for paired samples, when appropriate, was used to examine statistical significance of differences related to ADP concentration, exposure time, or the presence of indomethacin. For simplicity, SDs have been excluded from Figs 1 through 3; however, values for 1 SD are included in the legends.

Human subjects. Investigations were performed after approval by the local Human Investigation Committee and in accord with an assurance filed with and approved by the Department of Health and Human Services.

RESULTS

Platelet aggregation. Figure 1 shows the effect of ADP concentration on the time-course of aggregation during the first 5 seconds following stimulation. Control experiments, in which platelets were pumped through the apparatus with only saline as the "stimulating agent," revealed no significant shear-induced aggregation. Addition of 0.5 $\mu\text{mol/L}$ of ADP did not cause significant aggregation until 3 seconds, when $20.2\% \pm 12.8\%$ aggregation was observed. Higher concentrations of ADP induced significant aggregation by 0.3 seconds. Because differences in aggregation between 2 and 10 $\mu\text{mol/L}$ of ADP were insignificant at all time points, the aggregation trace for 2 $\mu\text{mol/L}$ of ADP was excluded from Fig 1.

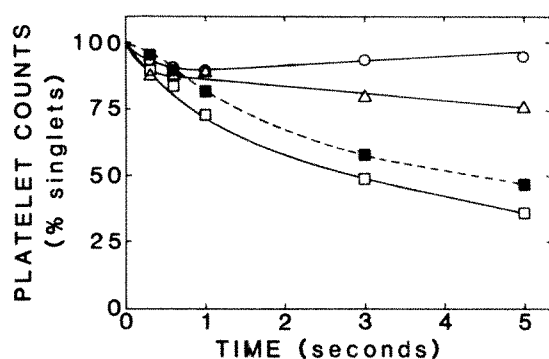


Fig 1. Influence of ADP concentration on early aggregation kinetics. [^{32}P]-Labeled platelet-rich Eagle's medium (PRE) was reacted with varying concentrations of ADP in the quenched-flow apparatus and quenched with glutaraldehyde (1% final concentration). The effluent at the various reaction times was collected, and samples were taken for platelet singlet counting to determine the extent of aggregation. In some experiments, the PRE was preincubated for 5 minutes with 1 $\mu\text{g/mL}$ of indomethacin to inhibit cyclooxygenase. The temperature was 37°C; the data are means from 15 control experiments, 7 experiments varying ADP from 0.5 to 10 $\mu\text{mol/L}$ of ADP and 8 experiments using 10 $\mu\text{mol/L}$ of ADP with and without indomethacin; SD ranged from 4.2% to 23.0% of the singlet count values, generally increasing with either ADP or time. The average platelet counts were 442,205/ μL (control) and 10 $\mu\text{mol/L}$ of ADP, 504,798/ μL (0.5 $\mu\text{mol/L}$ of ADP) and 387,437/ μL (10 $\mu\text{mol/L}$ of ADP with indomethacin). Concentrations of ADP in the reaction loop were (○), control (saline); (△), 0.5 $\mu\text{mol/L}$; (□), 10 $\mu\text{mol/L}$; and (■---), 10 $\mu\text{mol/L}$ (PRE preincubated with 1 $\mu\text{g/mL}$ of indomethacin).

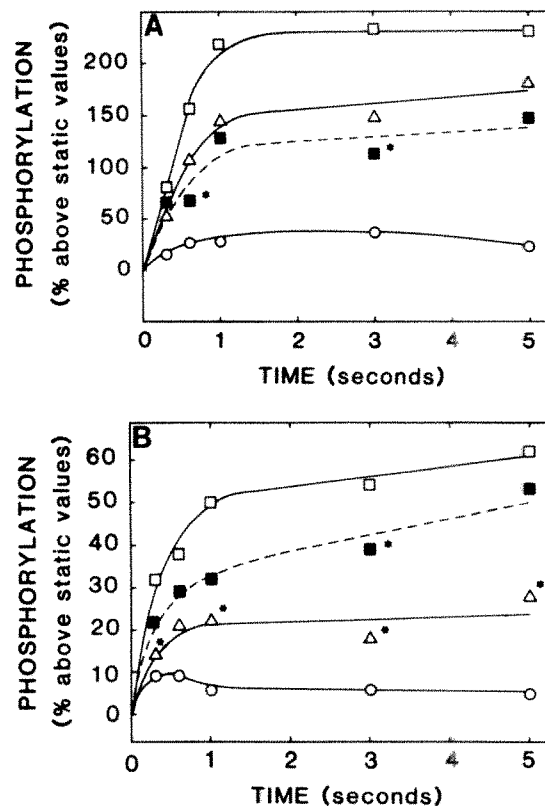


Fig 2. Influence of ADP concentration on early kinetics of phosphorylation of two platelet proteins: (A) the 20-K myosin light chain and (B) the 47-K protein. [^{32}P]-Labeled platelet-rich Eagle's solution (PRE) was reacted with varying concentrations of ADP in the quenched-flow apparatus and quenched with 3N perchloric acid. In some experiments, the PRE was preincubated for 5 minutes with 1 $\mu\text{g/mL}$ of indomethacin to inhibit cyclooxygenase. The temperature was 37°C. Protein samples from the quenched effluent were collected and electrophoresed as described in the Materials and Methods section. The data are derived from autoradiograms of the Coomassie-stained and dried gels that were scanned and whose various peaks were integrated and normalized for protein. The means are from 15 control experiments, 7 experiments varying ADP from 0.5 to 10 $\mu\text{mol/L}$ and 8 experiments using 10 $\mu\text{mol/L}$ of ADP with and without indomethacin. Data are expressed as the percentage of increase in phosphorylation over the value obtained from platelets not subjected to quenched-flow or exposure to ADP; SD ranged from 12% to 59% of the normalized phosphorylation values, generally increasing with either ADP or time. Platelet counts were the same as in Fig 1. Concentrations of ADP in the reaction loop were (○), control (saline); (△), 0.5 $\mu\text{mol/L}$; (□), 10 $\mu\text{mol/L}$; and (■---), 10 $\mu\text{mol/L}$ (PRE preincubated with 1 $\mu\text{g/mL}$ of indomethacin).

Protein phosphorylation. ADP increased myosin light chain (20-kD mol wt) phosphorylation and, under some circumstances, that of a 47-K protein (apparent mol wt 44 K in our system) phosphorylated by Ca^{2+} -activated phospholipid-dependent protein kinase C.¹ Both phosphorylations appear transitory since neither is apparent at 1 minute.¹⁸

We examined changes in phosphorylation of these two proteins during the first 5 seconds following stimulation using varying concentrations of ADP (0.5 to 10 $\mu\text{mol/L}$).

Figures 2A and B show the progress of these phosphorylations from 0.3 to 5 seconds after stimulation. With 0.5

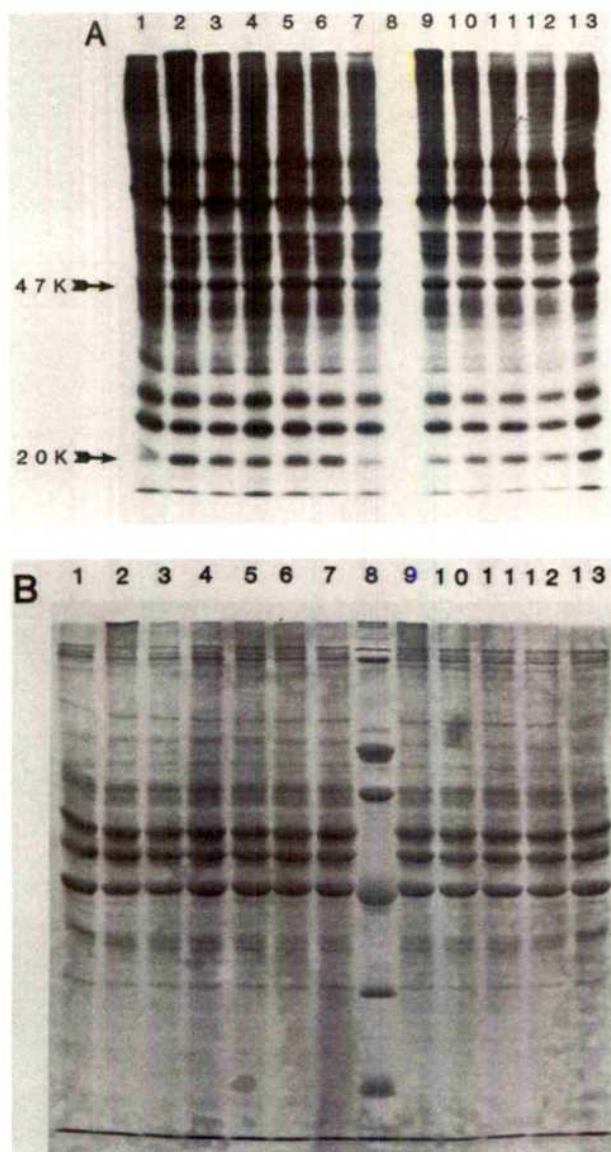


Fig 3. Autoradiogram and slab gel of [^{32} P]-labeled platelets showing the effect of indomethacin on ADP-induced protein phosphorylation. When data were interpreted, autoradiographic densities were always normalized for protein as described in the Materials and Methods section. (A) Autoradiograms. Lane 1: untreated (static) platelets. Lanes 2 through 6: platelets subjected to quenched-flow with 10 μ mol/L for 3, 5, 0.3, 0.6, or 1 second, respectively. Lane 7: platelets preincubated with 1 μ g/mL of indomethacin and not treated further. Lane 8 contained standard mol wt marker proteins (unlabeled). Lanes 9 through 13: platelets preincubated with 1 μ g/mL of indomethacin and subjected to quenched-flow with 10 μ mol/L ADP for 0.3, 0.6, 1, 3, or 5 seconds, respectively. (B) Coomassie-stained slab gel from which the autoradiogram was derived. Mol wt markers (lane 8) were 21,500, 31,000, 45,000, 66,200, 92,500, 116,250, and 200,000 daltons.

μ mol/L of ADP, increases in phosphorylation of the 20-K protein (Fig 2A) became significantly greater than control ($P < .05$) by 0.3 seconds, when it was 1.52 ± 0.45 times the static value, and continued to rise until at 5 seconds it was 2.81 ± 0.59 times the static value. Phosphorylation of the

47-K protein increased more slowly, reaching a level significantly above the static value at 0.6 seconds (1.21 ± 0.11 times the static, $P < .025$) but rising to only 1.28 ± 0.07 times the static at 5 seconds.

ADP 2 μ mol/L caused increased (2.41 ± 1.27 times the static, $P < .005$) phosphorylation of MLC at 0.3 seconds. Phosphorylation peaked at 3.78 ± 1.22 times static at 3 seconds and then fell an insignificant amount to 3.66 ± 1.00 times the static at 5 seconds; 47-K phosphorylation was also increased at 0.3 seconds (1.36 ± 0.34 times static, $P < .025$) and continued to increase very slowly, as with 0.5 μ mol/L of ADP, reaching a level 1.66 ± 0.31 times static at 5 seconds.

ADP 10 μ mol/L, caused increases in 20-K and 47-K phosphorylation very similar to that which occurred with 2 μ mol/L of ADP. There were no significant differences, and only the time-course for 10 μ mol/L of ADP is shown.

Dose-response relationship for aggregation and phosphorylation. The concentration of ADP required to give half-maximal aggregation or phosphorylation (K_a) was derived from the dose-response curves for the various reaction time points. The average concentration required to give a half-maximal response was 0.7 ± 0.2 μ mol/L of ADP for 20-K phosphorylation, 1.0 ± 0.1 μ mol/L of ADP for 47-K phosphorylation, and 1.2 ± 0.3 μ mol/L of ADP for aggregation. The K_a for 20-K phosphorylation was significantly less ($P < .025$) than that for 47-K phosphorylation and that for aggregation ($P < .01$). Table 1 shows the comparison between half-maximal concentrations for ADP and thrombin. The difference between the two was most evident when the half-maximal concentrations for aggregation were compared with those for 47-K phosphorylation. Although aggregation is more sensitive to thrombin than is 47-K phosphorylation,⁵ it was less sensitive to ADP. Moreover, although the half-maximal concentrations of thrombin for 20-K and aggregation are almost identical, they were significantly different for ADP.

Effect of Indomethacin. In a separate series of experiments, to test for potential influences of cyclooxygenase derived metabolites, the PRE was incubated for 5 minutes with 1 μ g/mL of indomethacin before exposure to 10 μ mol/L of ADP. Indomethacin did not affect aggregation (Fig 1), but phosphorylation of the 47-K protein was partially inhibited, although only significantly at one time point. Indometh-

Table 1. Comparison of Half-Maximal Response Doses for ADP and Thrombin

Response	K_a (μ mol/L ADP)	K_a (U/mL Thrombin)
Aggregation	$1.2 \pm 0.3^*$	0.51 ± 0.18
20-K Phosphorylation	0.7 ± 0.2	0.53 ± 0.16
47-K Phosphorylation	$1.0 \pm 0.1^\dagger$	$1.10 \pm 0.44^\ddagger$

Data are derived from dose-response curves at 0.3, 0.6, 1, 3, and 5 seconds for 15 experiments using thrombin and 15 experiments using ADP. They represent the average $\pm$ SD dose required to give half-maximal response.

*Significantly greater than K_a for 20-K phosphorylation, $P < .01$.

† Significantly greater than K_a for 20-K phosphorylation, $P < .025$.

‡ Significantly greater than K_a for aggregation on 20-K phosphorylation, $P < .25$.

acin did significantly inhibit the increase in myosin light chain phosphorylation by ~40% ($P < .05$). Figure 3 shows an autoradiograph and the corresponding Coomassie-stained gel from one of these experiments.

DISCUSSION

Effective hemostasis *in vivo* requires that platelets respond very rapidly to a physiologic activating challenge. Activation times as short as 2 milliseconds have been suggested.^{6,7} Therefore, an understanding of the earliest morphological and biochemical events in platelet stimulus-response and how they are interrelated is important.

The immediate platelet biochemical response to a particular aggregating agent may involve any, but not necessarily all, of the following: (a) hydrolysis of metabolic ATP¹⁹; (b) changes in cytoplasmic pH and membrane potential,²⁰ perhaps as a result of Na^+/H^+ exchange across the membrane²¹; (c) inhibition (or activation) of adenyl cyclase and cAMP-dependent protein kinase²²; (d) activation of phospholipase C, resulting in a rapid turnover of phosphoinositides, production of inositol phosphates and diacylglycerol, and activation of protein kinase C²³; (e) increased cytoplasmic calcium levels²⁴ (from extracellular sources and/or release from the platelet dense tubular system) and activation of Ca^{2+} /calmodulin-dependent enzymes including a protease²⁵ and myosin light chain kinase; and (f) coalescence and/or dispersal of various components of the platelet contractile system.²⁵

The order of these events and the degree of completion of the reactions involved may vary with the aggregating agent and its concentration. Morphological changes such as shape change, aggregation, and granule centralization, labilization, and secretion may also be dependent on these variations.

Thus thrombin, which raises intracellular Ca^{2+} and activates phospholipase C (producing the protein kinase C activator diacylglyceride), causes the phosphorylation of MLC and of the 47-K protein to about the same extent in the first 5 seconds of response.⁵ In the present study ADP, which also raised intracellular Ca^{2+} but may or may not activate phospholipase C,^{8,26} was used as the aggregating agent. As might be predicted from the dependence of protein kinase C activity on diacylglyceride, ADP caused much more extensive phosphorylation of MLC than of the 47-K protein, and less increase in 47-K phosphorylation than thrombin. Indeed, ADP increased MLC phosphorylation approximately twice as much as thrombin. Both phosphorylations were essentially complete at 1 sec v 3 seconds for thrombin, suggesting that the initial rise in intracellular Ca^{2+} may occur earlier and be greater with ADP than with thrombin. Preliminary experiments in our laboratory using the fluorescent Ca^{2+} probe, Indo-1, indicate that the ADP-induced increase in intracellular Ca^{2+} is indeed faster, and that the level of Ca^{2+} is higher with ADP than with thrombin during the first 5 seconds after stimulation.²⁷ Sage and Rink,⁹ using Fura-2, showed that

ADP induced an earlier increase in internal Ca^{2+} than did thrombin.

A major difference between the two aggregating agents is evident when half-maximal concentrations for aggregation and the two phosphorylations are compared (Table 1): 20-K phosphorylation was more sensitive than 47-K phosphorylation for both aggregating agents. The K_a for thrombin-induced aggregation was almost identical to that of 20-K phosphorylation, however, whereas the K_a for ADP-induced aggregation was significantly greater than that of ADP-induced 20-K phosphorylation. Moreover, although aggregation is more sensitive to thrombin than is 47-K phosphorylation, it is not more sensitive to ADP. Thus, any direct connection between aggregation and either of the two phosphorylations cannot be valid for all aggregating agents. MLC phosphorylation is essential for the ATPase activity and force generation in platelet actomyosin²⁷ and is associated with MLC incorporation into the platelet cytoskeleton.²⁸ The phosphorylation of MLC may be an essential prerequisite to platelet aggregation, perhaps by playing a part in the exposure of fibrinogen receptors. There appears to be another prerequisite as well, however, one that may be fulfilled prior to MLC phosphorylation with thrombin, but after it with ADP.

Our previous study showed that thrombin-induced MLC phosphorylation is partially dependent on Ca^{2+} from external sources.⁵ The present study shows that ADP-induced MLC and 47-K phosphorylation may also be partially dependent on thromboxane A_2 formation, as they were inhibited by indomethacin. Phosphorylation of MLC was more inhibited than 47-K phosphorylation. Because thromboxane A_2 activates phospholipase C,⁸ inhibition of its production might be expected to cause a greater reduction in 47-K phosphorylation than in MLC phosphorylation. One possible explanation of our results may be that ADP-induced activation of protein kinase C is entirely due to increased cytoplasmic Ca^{2+} .²⁹ When thromboxane A_2 production is inhibited, arachidonic acid may be redirected into the production of inhibitory leukotrienes,³⁰ which have been suggested to interfere with the rise in cytoplasmic calcium.³¹ In this case, since MLC phosphorylation is more sensitive to Ca^{2+} than is 47-K phosphorylation,³⁰ MLC phosphorylation would suffer a greater reduction.

These studies provide new evidence that early biochemical events in the stimulus-response sequence of blood platelets may vary according to the stimulus in ways directly relevant to platelet function. The time-scale approaches that needed for efficient hemostasis *in vivo*^{6,7} and complements our earlier studies of shape-change, aggregation, and thrombin-induced protein phosphorylation.²⁻⁵ It will be instructive to correlate cytoskeleton dynamics, Ca^{2+} movements, phospholipid turnover, and the activities of phospholipases with phosphorylation and functional phenomena and to compare these among other aggregating agents as well as thrombin and ADP.

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Effects of Herpes Virus Carrier Status on Peripheral T Lymphocyte Subsets

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We studied the effects of herpes virus carrier status on peripheral blood T lymphocyte subsets in 334 healthy individuals. IgG-class antibodies against cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), and varicella-zoster virus (VZV) were used as markers for the carrier status of those viruses. CMV carrier status was associated with significant increases in the numbers of some T cell subsets, whereas the carrier status of EBV, HSV, and VZV had no significant effects. The 159 CMV-seropositive individuals had higher numbers of HNK1+ T cells than did the 175 CMV-seronegative individuals [mean (SD), 292 (196)/ μ L v 164 (89)/ μ L, respectively], including the CD4+HNK1+ T cells [38 (48)/

μ L v 9 (13)/ μ L, respectively] and the CD8+HNK1+ T cells [166 (146)/ μ L v 73 (54)/ μ L, respectively]. Morphological and cytochemical studies showed that the expression of HNK1 by the CD4+ and CD8+ T cells was associated with the occurrence of azurophilic cytoplasmic granules and a loss of nonspecific esterase activity. The numbers of CD4+HNK1+ and CD8+HNK1+ T cells increased proportionally to the levels of the IgG-class CMV antibody titers. We suggest that the increased numbers of CD4+HNK1+ and CD8+HNK1+ granular T cells in CMV carriers reflect the persistent interaction between CMV and the immune system of its hosts.

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AN IMPORTANT characteristic of the herpes group viruses is their ability to persist in the tissues of their hosts for many years after the initial infection. Consequently, the prevalence of antibodies against those viruses increases with age.¹ Acute infection with cytomegalovirus (CMV) or Epstein-Barr virus (EBV) influences significantly the numbers of peripheral T lymphocytes, particularly those expressing the CD8 marker.^{2,3} Such increases in CD8+ T cells have also been reported in recipients of renal⁴ and bone marrow⁵ allografts. Active CMV infection is associated with a persistent increase of CD8+ T cells that also express the HNK1 antigen in recipients of cardiac⁶ and bone marrow⁷ allografts. The HNK1 marker was originally described as occurring on large granular lymphocytes with natural killer (NK) function.⁸

The number of T cells in the peripheral blood decreases with increasing age,⁹⁻¹¹ but the subsets of T cells that coexpress CD4 or CD8 and HNK1 increase in number.¹² That situation may result from the increased numbers of viruses that the elderly carry as compared with younger individuals. We investigated whether the herpes virus carrier status of healthy individuals influences significantly their peripheral blood T cell subsets, particularly those coexpressing the HNK1 marker.

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MATERIALS AND METHODS

Healthy volunteer blood donors. Three hundred thirty-four healthy individuals (153 males and 181 females) participated in the study. Their age distribution is shown in Table 1. Of those 334 individuals, 131 were blood donors of the Dutch Red Cross Blood Transfusion Service, 171 were hospital personnel (University Hospital, Leiden, The Netherlands) and 32 were relatives or friends of the hospital staff.

Isolation and fractionation of mononuclear cells. Heparinized venous blood samples were drawn between 8 and 11 AM to exclude the influence of circadian variations on T cell subsets.¹³ The mononuclear cells were isolated using Ficoll-Isopaque density-gradient centrifugation and enriched for T lymphocytes using 2-aminoethyl-isothiourea-bromide hydrobromide (AET) pretreated sheep RBCs (SRBCs).¹⁴ The proportion of cells that formed rosettes with the AET-pretreated SRBCs was counted among 200 mononuclear cells of each sample using phase-contrast microscopy. The SRBC-rosetting cells are referred to as T cells in the following sections. After the SRBCs were lysed the T cell-enriched fractions were washed twice in phosphate-buffered saline containing 1% bovine serum albumin (PBS/BSA) and resuspended in RPMI 1640 medium containing 10% fetal calf serum (RPMI/FCS) until further use.

Prior to cell sorting, the T cells were incubated with the following mixtures of monoclonal antibodies (MCAs) that were conjugated either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): anti-Leu 3a/PE (CD4) + anti-Leu 7/FITC (anti-HNK1) and anti-Leu 2a/PE (CD8) + anti-Leu 7/FITC for 30 minutes at 4 °C. After being washed once in PBS/BSA, the T cells were resuspended in RPMI/FCS and fractionated into CD4+HNK1+ and CD4+HNK1- or CD8+HNK1+ and CD8+HNK1- subsets using a FACS (FACS IV; Becton Dickinson, Mountain View, CA) equipped with an argon-ion laser tuned at 488 nm with an intensity of 300 mW. Fluorescent signals for each fluorochrome were detected selectively using a 530-nm band pass filter for green (FITC) emission and a 575-nm band pass filter for red-orange (PE) emission. Cells other than living T cells were electronically eliminated on the basis of forward and right-angle light scatter. The sorted cells were collected in plastic tubes prefilled with RPMI/FCS.

Analysis of T cell subsets. The T cell subsets are described using the CD nomenclature of the Third International Workshop and Conference on Human Leukocyte Differentiation Antigens (Oxford, England, September 21-26, 1986), except for the HNK1+ and NK1+ subsets, for which no Workshop nomenclature is

Table 1. Prevalence of Herpes Virus Antibodies

Age Category (yr)	n	Prevalence of Viral Antibodies*			
		CMV	EBV	HSV	VZV
5-20	24	38	67	33	88
21-30	126	42	82	50	97
31-40	107	52	82	69	99
41-50	43	56	86	72	100
51-60	22	41	91	68	100
61-64	12	67	100	100	100

Abbreviations: HSV, herpes simplex virus; VZV, varicella-zoster virus.

*Percentage of seropositive individuals.

available. Therefore, we refer to those subsets using the nomenclature given by original investigators.^{15,16}

The following MCAs were used: anti-Leu 4 (CD3); anti-Leu 3a (a CD4 MCA of the IgG₁ subclass); FK18 (a CD8 MCA of the IgG₃ subclass); anti-Leu 7 (an anti-HNK1 MCA of the IgM class); anti-Leu 19 (an anti-NKH1 MCA of the IgG₁ subclass); and anti-HLA-DR (an anti-class II backbone MCA of the IgG_{2a} subclass). The coexpression of HNK1 by CD4+, CD8+, and NKH1+ T cells and the degree of HLA-DR expression by HNK1+ T cells was studied using two-color immunofluorescence. For those purposes, the HNK1 marker was identified using anti-Leu 7 conjugated with biotin (B), and the T cells were incubated with the following mixtures: anti-Leu 3a + anti-Leu 7/B; FK18 + anti-Leu 7/B; anti-Leu 19 + anti-Leu 7/B; and anti-HLA-DR + anti-Leu 7/B. In the second step of the single-color immunofluorescence analyses, goat-anti-mouse Ig (GAM) conjugated with FITC was used. In the second step of the two-color immunofluorescence analyses, the appropriate mixtures of streptavidin conjugated with PE and class-specific and subclass-specific GAM antibodies conjugated with FITC were added to the T cells. All incubations were for 30 minutes at 4 °C. The anti-Leu MCA and the streptavidin were purchased from Becton Dickinson; FK18 was purchased from the National Institute of Public Health and Environmental Hygiene (Bilthoven, The Netherlands), and all GAM antibodies were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). Flow cytometry was performed using forward and right-angle light scatter on the FACS IV. For single-color immunofluorescence, histograms were recorded for 10,000 T cells per sample and for two-color immunofluorescence, 5,000 HNK1+ T cells were analyzed per sample.

The absolute numbers of the different T cell subsets were calculated from the percentages obtained by flow cytometry and from the simultaneously obtained absolute T cell counts. The latter were calculated from the percentages of SRBC-rosetting cells among the mononuclear cells and the absolute numbers of mononuclear cells as determined with a Hemalog D cell counter (Technicon, Tarrytown, NY).

Morphological studies. The sorted T cells were washed once with PBS/BSA, and cytocentrifuge preparations were made. The slides were stained with May-Grünwald Giemsa (MGG) and for acid phosphatase¹⁷ and nonspecific esterase¹⁸ [α -naphthyl-acetate-esterase (ANAE), and α -naphthylbutyrate-esterase (ANBE)] activities.

For ultrastructural studies, a pellet containing at least 0.5×10^6 sorted T cells was washed with PBS containing 0.042% KCl and 0.025% CaCl₂. The cells were fixed for 10 minutes at room temperature in 1.5% glutaraldehyde in 0.14 mol/L of cacodylate buffer (pH 7.4). Thereafter, the cells were washed in PBS and finally fixed in 1% OsO₄ in 0.14 mol/L of cacodylate buffer (pH 7.2) for 60 minutes at 4 °C. Dehydration, embedding, and sectioning were done according to standard techniques. After being stained briefly with uranyl-acetate and leadhydroxide, the sections were studied with an EM 300 or EM 410 electron microscope (Philips, Eindhoven, The Netherlands).

Detection of herpes virus antibodies. IgM-class and IgG-class antibodies to the late antigens of CMV were detected using an enzyme-linked immunosorbent assay (ELISA).¹⁹ Antibody titers of ≥ 40 in the IgG assay and of ≥ 200 in the IgM assay were considered positive.

IgG-class antibodies to herpes simplex virus (HSV), to varicella-zoster virus (VZV), and to the capsid antigen of EBV were detected using indirect immunofluorescence. Human embryonic lung fibroblasts were infected with an HSV-2 strain or a VZV strain. P3HR₁ cells were cultured for the production of EBV capsid antigen. Smears of the infected antigen-bearing cells and the noninfected control cells were made on slides, which were then fixed by acetone and methanol. Before testing, nonspecific fluorescent factors were removed from the test sera by absorption.²⁰ Dilutions were made in veronal-buffered saline containing 10% fresh guinea pig serum. After incubation and washing, the slides were treated with sheep anti-human IgG conjugated with FITC (prepared by Dr J. Nagel, National Institute of Public Health and Environmental Hygiene). Positive and negative sera were used as controls. Antibody titers of ≥ 16 (HSV and VZV) or of ≥ 32 (EBV) were considered positive.

Table 2. Influence of Age and Gender on T Cell Subsets

T Cell Subset	Age Categories (yr)†			Correlation Coefficient*	Gender‡	
	5-30 (n = 150)	31-50 (n = 150)	51-64 (n = 34)		M (n = 153)	F (n = 181)
CD3+	1,378	1,339	1,133	-0.16	1,347 (651, 2,423)	1,328 (608, 2,454)
CD4+	845	845	777	-0.08	844 (399, 1,554)	836 (388, 1,837)
CD8+	465	437	369	-0.21	435 (184, 1,090)	432 (182, 776)
HNK1+	167	195	242	+0.16	208 (67, 591)	168 (62, 405)
CD4+HNK1+	7	11	18	+0.18	13 (<5, 109)	8 (<5, 84)
CD4+HNK1-	816	814	709	-0.10	812 (395, 1,496)	811 (376, 1,807)
CD8+HNK1+	81	97	117	+0.12	107 (12, 334)	82 (16, 274)
CD8+HNK1-	363	328	246	-0.29	315 (128, 800)	347 (110, 664)

*Spearman's rank correlation test. A correlation coefficient $> +0.11$ or < -0.11 differed significantly from 0 at the $P < .05$ level.

†Median number of T cells per microliter.

‡ $P = .002$ (Wilcoxon's test).§ $P = .01$ (Wilcoxon's test).

|| Median (5th and 95th percentiles) number of T cells per microliter.

Table 3. CMV and EBV Carrier Status and T Cell Subsets

T Cell Subset	CMV Seropositive* (n = 175)	CMV Seronegative* (n = 159)	EBV Seropositive* (n = 283)	EBV Seronegative* (n = 51)
CD3+	1,382 (763, 2,681)	1,265 (523, 2,291)	1,319 (647, 2,421)	1,436 (533, 2,524)
CD4+	877 (457, 1,840)	814 (354, 1,541)	826 (391, 1,636)	888 (372, 1,690)
CD8+	470 (231, 1,096)	415 (171, 776)	430 (210, 865)	454 (169, 1,026)
HNK1+	237 (83, 629)	149 (56, 342)	194 (63, 528)	167 (64, 436)
CD4+HNK1+	23 (<5, 150)	7 (<5, 31)	11 (<5, 107)	5 (<5, 77)
CD4+HNK1-	846 (452, 1,814)	794 (351, 1,525)	801 (382, 1,619)	870 (367, 1,680)
CD8+HNK1+	136 (21, 332)	66 (12, 185)	95 (15, 285)	66 (9, 335)
CD8+HNK1-	331 (123, 750)	342 (124, 710)	327 (128, 696)	404 (114, 808)

P values were determined using standard regression analyses that controlled for age, gender, and carrier status of CMV, EBV, HSV, and VZV (described in the Materials and Methods section). Only *P* values <.05 are stated.

*Median (5th and 95th percentiles) number of T cells per microliter.

†*P* = .0002.

‡*P* value range .001 to .01.

§*P* ≤ .0001.

|| *P* value range .02 to .05.

Statistical analyses. Because the T cell subset data were in general not normally distributed, nonparametric methods were used for univariate statistical analyses, as indicated. The effects of herpes virus carrier status on T cell subsets was studied using multivariate analyses, prior to which the T cell subset data were transformed to their natural logarithms to reduce skewness. For multivariate analyses, standard linear regression procedures were used that controlled for potentially confounding factors, as indicated.

RESULTS

Prevalence of herpes virus antibodies. The prevalence of herpes virus antibodies against CMV, EBV, HSV, and VZV in relation to the ages of the 334 individuals is shown in Table 1. Prevalence of antibodies increased against all four viruses with increasing age and was most evident for HSV. Of the 159 individuals who were seropositive for IgG-class CMV antibodies, 19 were also seropositive for IgM-class CMV antibodies. All 175 individuals who were seronegative for IgG-class CMV antibodies were also seronegative for IgM-class CMV antibodies.

Influence of age and gender on T cell subsets. Because age and gender influence some lymphocyte subpopulations significantly, particularly HNK1+,^{12,15} we examined the effects of those parameters on the T cell subset counts to

correct for their possible confounding effects in a multivariate statistical model. The significant influences of age and gender on T cell subsets as analyzed using univariate statistics are shown in Table 2. With increasing age, the CD3+ and CD8+ T cells decreased in number, whereas the HNK1+ T cells increased in number. As a result, the CD4+HNK1+ and CD8+HNK1+ T cells increased, whereas the CD8+HNK1- T cells (constituting the majority of CD8+ cells) decreased in number. Males had significantly higher numbers of HNK1+ and CD8+HNK1- T cells than did females. Consequently, we decided to analyze the influence of the herpes virus carrier status on T cell subsets using multivariate statistical models that also controlled for age and gender. In those analyses, the above-described effects of age and gender on T cell subsets and their significance were confirmed (data not shown).

Influence of herpes virus carrier status on T cell subsets. The effects of CMV and EBV carrier status on T cell subsets are shown in Table 3; those of HSV and VZV are shown in Table 4. The CD3+, CD4+, CD8+, and especially the HNK1+ T cell subsets were all significantly increased in number in the CMV-seropositive group as compared with the CMV-seronegative group. Analysis of the coexpression of HNK1 by the CD4+ and CD8+ T cells revealed that the

Table 4. HSV and VZV Carrier Status and T Cell Subsets

T Cell Subset	HSV Seropositive* (n = 203)	HSV Seronegative* (n = 131)	VZV Seropositive* (n = 326)	VZV Seronegative† (n = 8)
CD3+	1,297 (619, 2,508)	1,363 (615, 2,421)	1,328 (635, 2,425)	1,446 (544, 3,323)
CD4+	817 (386, 1,660)	848 (402, 1,577)	836 (390, 1,647)	882 (346, 1,555)
CD8+	415 (185, 912)	464 (183, 885)	433 (190, 893)	512 (186, 1,379)
HNK1+	198 (69, 540)	171 (62, 464)	193 (65, 523)	151 (64, 460)
CD4+HNK1+	11 (<5, 111)	8 (<5, 71)	11 (<5, 104)	13 (<5, 66)
CD4+HNK1-	794 (372, 1,654)	828 (399, 1,568)	812 (385, 1,620)	837 (342, 1,540)
CD8+HNK1+	97 (21, 293)	80 (8, 282)	89 (15, 285)	68 (12, 149)
CD8+HNK1-	307 (113, 674)	373 (139, 738)	333 (125, 710)	448 (150, 1,296)

P values were determined using standard regression analyses that controlled for age, gender, and carrier status of CMV, EBV, HSV, and VZV (described in the Materials and Methods section). None of the *P* values reached the .05 significance level.

*Median (5th and 95th percentiles) number of T cells per microliter.

†Median (minimum and maximum) number of T cells per microliter.

Table 5. CMV Carrier Status and Expression of HNK1 and NKH1 by T Cells

T Cell Subset	CMV Seropositive* (n = 42)	CMV Seronegative* (n = 48)	P Value†
HNK1+	222 (65, 591)	137 (37, 421)	.004
NKH1+	127 (36, 382)	103 (28, 331)	NS‡
HNK1+NKH1+	54 (11, 222)	56 (11, 173)	NS‡
HNK1+NKH1-	132 (21, 385)	60 (6, 278)	.002

*Median (5th and 95th percentiles) number of T cells per microliter.

†P values were determined using standard regression analyses that controlled for age, gender, and CMV carrier status (described in the Materials and Methods section).

‡NS, P value > .05.

very few CD4+HNK1+ T cells and the larger subset of CD8+HNK1+ T cells were clearly and significantly increased in the CMV-seropositive group as compared with the CMV-seronegative group. There was also a slight increase in CD4+HNK1- T cells in the CMV-seropositive group as compared with the CMV-seronegative group that was of borderline significance ($P = .03$). The effects of EBV, HSV, and VZV carrier status on T cell subsets was not significant except for an EBV-associated increase in CD4+HNK1+ T cells that was of borderline significance ($P = .048$).

The relation between the expression of the HNK1 and NKH1 markers was studied on the T cells of 90 individuals (Table 5). The number of HNK1+ T cells lacking the NKH1 marker were significantly higher in 42 CMV-seropositive individuals than in 48 CMV-seronegative individuals ($P = .002$), whereas the numbers of T cells expressing both HNK1 and NKH1 were similar. The expression of HLA-DR was studied on the T cells of 32 individuals (12 CMV seropositive and 20 CMV seronegative). A median of only 2% (range 1% to 12%) of their T cells reacted with the anti-HLA-DR MCAs above background level. The seven

individuals who had $\geq 4\%$ of their T cells stained with anti-HLA-DR were all CMV seronegative. Almost no HNK1+ T cells reacted with anti-HLA-DR (range 1% to 3%).

We then studied the interactions between the levels of the IgG-class CMV antibody titers and the numbers of HNK1+, CD4+HNK1+, and CD8+HNK1+ T cells. The numbers of CD8+HNK1+ T cells are plotted against the IgG-class CMV antibody titers in Fig 1. We analyzed those correlations in the 159 CMV-seropositive individuals with multivariate statistics that controlled for age, gender, IgG-class CMV antibody titers, and the presence or absence of IgM-class CMV antibodies. The analyses confirmed the significance of those correlations (HNK1+ T cells, $P = .01$; CD4+HNK1+ T cells, $P = .003$; and CD8+HNK1+ T cells, $P = .003$). The influence of IgM-class CMV antibodies on those parameters was not significant.

Morphological studies of sorted T cell subsets. We examined the morphological and cytochemical characteristics of the CD4+ and CD8+ T cells of three CMV-seropositive individuals in relation to the HNK1 expression by those cells (Table 6). They were selected because of their relatively high proportions of T cells expressing HNK1. Staining of the sorted T cell subsets with MGG revealed that the majority of CD4+HNK1+ and CD8+HNK1+ T cells had azurophilic granules scattered through moderate to large amounts of cytoplasm. The CD4+HNK1- T cells had scarce amounts of cytoplasm and were generally agranular. Also, the majority of CD8+HNK1- T cells had small amounts of cytoplasm without granules, but in 20% to 26% of these cells more cytoplasm was present containing several azurophilic granules.

After staining was done for nonspecific esterase activity, a dotlike staining pattern was observed in a sizable proportion of CD4+HNK1- and CD8+HNK1- T cells, whereas only a minority of CD4+HNK1+ and CD8+HNK1+ T

Fig 1. Interaction between the numbers of peripheral blood CD8+HNK1+ T cells and the levels of IgG-class cytomegalovirus (CMV) antibody titers in 334 healthy individuals. Each point in the figure represents one individual. A logarithmic scale has been used for the vertical axis to compress the figure.

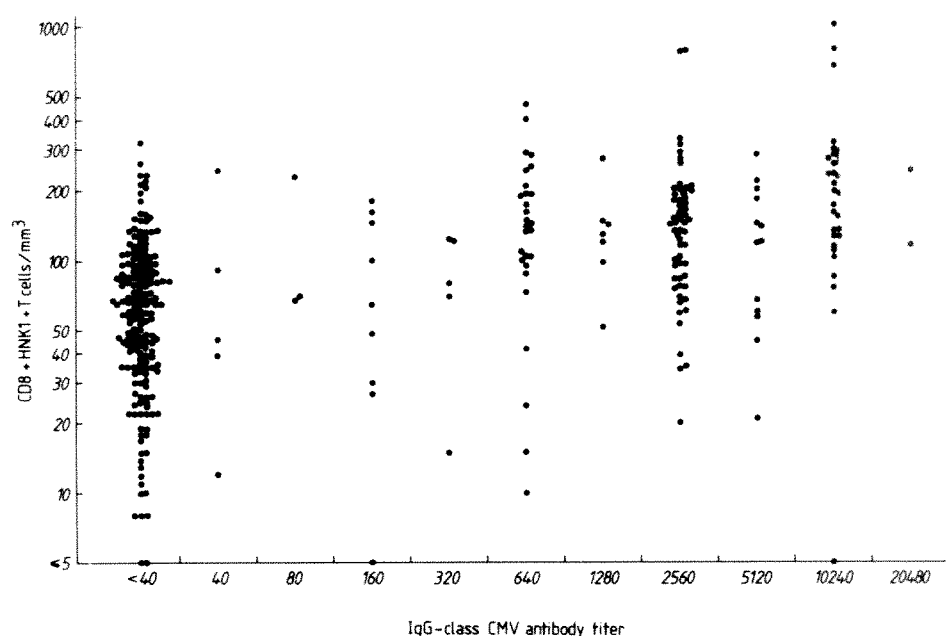


Table 6. Morphological Characteristics of CD4+ and CD8+ T Cells in Relation to HNK1 Expression

T Cell Subset	May-Grünwald Giemsa Cytoplasmatic Granules*	Nonspecific Esterase Activity*	
		Dotlike Staining	Granular Staining
CD4+HNK1+	73, 78, 74	13, 11, 11	2, 1, 7
CD4+HNK1-	2, 5, 3	ND, 51, 54	ND, 6, 3
CD8+HNK1+	89, 93, 85	2, 5, 9	0, 2, 2
CD8+HNK1-	21, 20, 26	ND, 54, 38	ND, 1, 2

Abbreviation: ND, not done.

*Percentage of cells in three CMV seropositive individuals. Results obtained in those individuals are presented in the same order throughout.

cells did so (Table 6). A granular staining pattern for nonspecific esterase activity was apparent in <10% of all four sorted T cell subsets. Staining for ANAE and ANBE activities yielded similar results (data not shown). Cytochemical acid phosphatase localization revealed that >95% of all four sorted T cells subsets were positive.

The ultrastructural characteristics of the CD4+ and CD8+ T cells of two CMV-seropositive individuals were studied in relation to the expression of HNK1 by those cells (Figs 2 and 3). The CD4+HNK1+ and CD8+HNK1+ T cells generally had an extended cytoplasm as compared with the CD4+HNK1- and CD8+HNK1- T cells, and they often had an irregularly shaped nucleus. The cytoplasm of the HNK1+ T cells contained heterogeneous electron-dense bodies, which were somewhat more numerous in the CD8+HNK1+ T cells than in the CD4+HNK1+ T cells.

The Golgi apparatus of those cells was moderately developed.

DISCUSSION

CMV carrier status of healthy individuals is associated with significant increases in number of some peripheral blood T cell subsets, particularly those expressing the HNK1 marker. Those T cells also express CD4 or CD8 but not NKH1 and have the morphological, cytochemical, and ultrastructural characteristics of granular lymphocytes.

The interaction between CMV carrier status and T cell subsets is independent of age and gender. We noted similar changes in T cell subsets with increasing age, as reported by other investigators, ie, decreases in CD3+, CD4+, CD8+, and CD8+HNK1- T cell numbers and increases in HNK1+, CD4+HNK1+, and CD8+HNK1+ T cell numbers.^{11,12,15,21} Our observation that males have higher numbers of HNK1+ T cells is in accordance with a previous report.¹⁵

The significance of the effects of herpes virus carrier status on T cell subsets appears to be limited to CMV, since those effects are not significant for EBV and HSV. One should be cautious in assessing the significance of the effect of the VZV carrier status on T cell subsets because of the very low number of VZV-seronegative individuals in our study group. The positive correlations between the levels of IgG-class CMV antibody titers and the numbers of HNK1+, CD4+HNK1+, and CD8+HNK1+ T cells may indicate that the magnitude of the CMV-specific immune

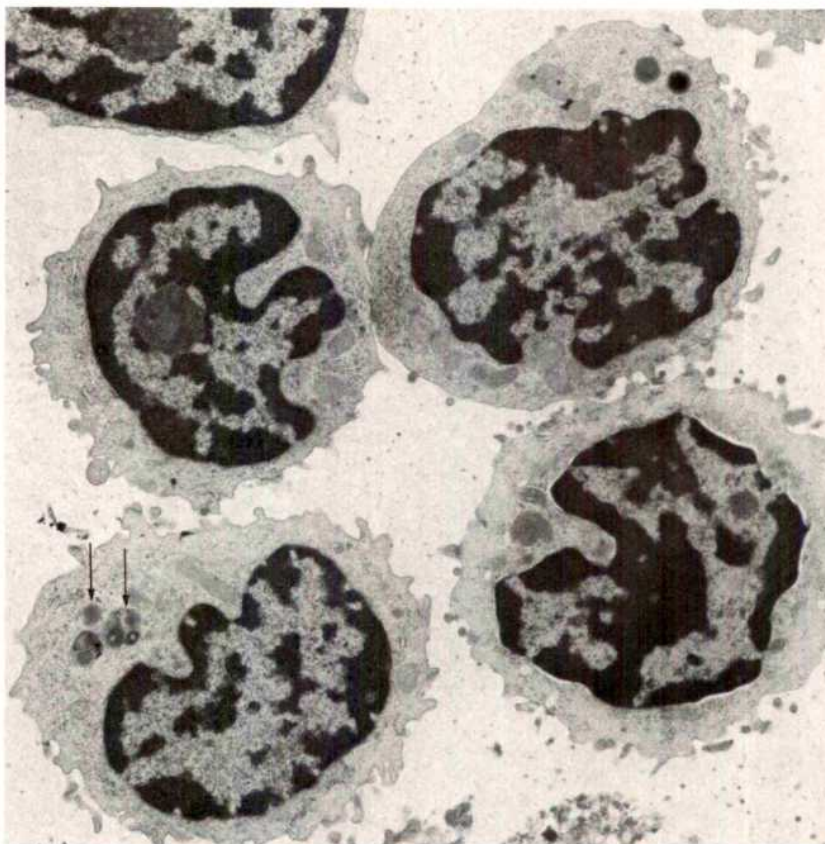


Fig 2. Ultrastructural characteristics of CD4+HNK1+ T cells. The nuclei have an irregular shape. Dense bodies are apparent in the cytoplasm of two CD4+HNK1+ T cells (arrows). Original magnification $\times 9,400$; current magnification $\times 6,580$.

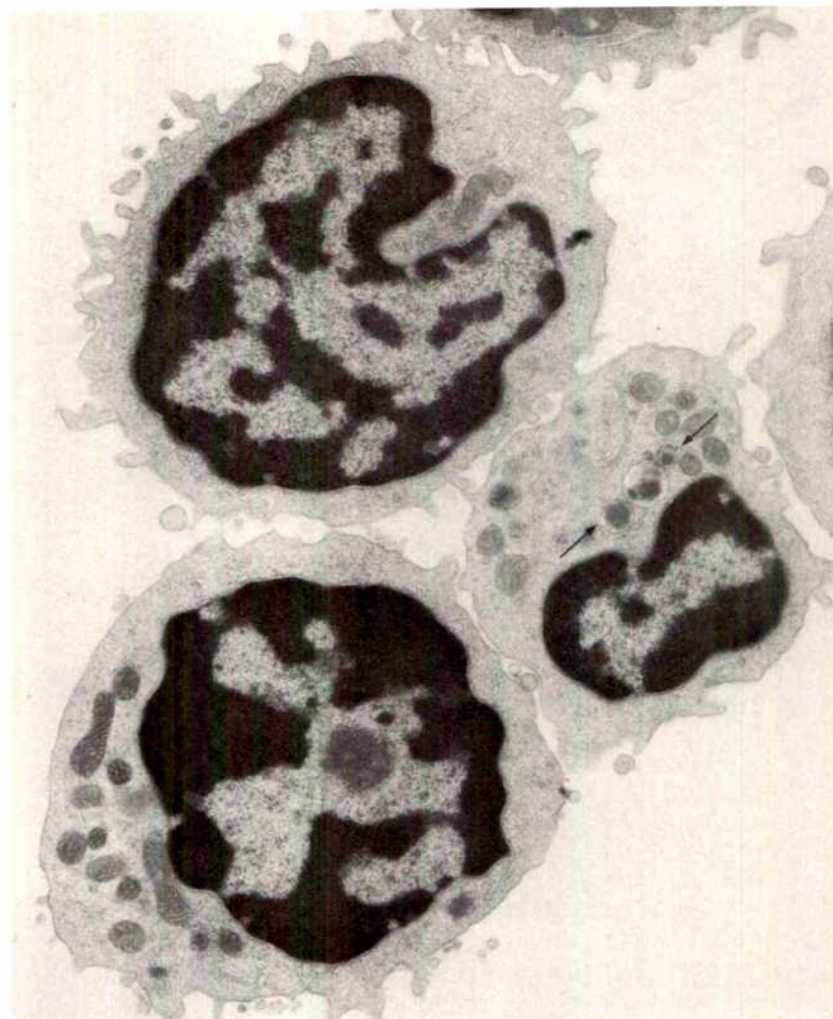


Fig 3. Ultrastructural characteristics of CD8+HNK1+ T cells. Dense bodies (arrows) and the Golgi apparatus are apparent in one cell. Original magnification $\times 11,900$; current magnification $\times 8,330$.

response relates to the HNK1 expression by peripheral T cells.

The positive correlation between age and HNK1 expression by peripheral blood T cells suggest that antigenic stimuli other than CMV may also be involved. A marked and significant increase in CD8+HNK1+ lymphocytes has been reported in patients with the acquired immunodeficiency syndrome (AIDS), the AIDS-related complex of clinical signs,^{22,23} and also in patients treated with clotting factor concentrates for their congenital coagulation defects.²⁴ Those increases in CD8+HNK1+ lymphocytes may have been due to infection with the human immunodeficiency virus, but also to chronic exposure to CMV or other antigenic stimuli present in semen or clotting factor concentrates. Increased numbers of CD4+HNK1+ and CD8+HNK1+ lymphocytes have been reported in long-term survivors after allogeneic kidney,²⁵ heart,⁶ or bone marrow transplants (BMTs)^{7,26} and correlated with CMV infection.^{6,7} The results are all consistent with the hypothesis that the increased numbers of HNK1+ T cells represent an immune response to chronic antigenic stimulation.

Interestingly, the occurrence of increased proportions of peripheral blood HNK1+ granular lymphocytes coexpressing CD4 or CD8 has been reported in patients with B cell

malignancies.²⁷⁻²⁹ However, the majority of the CD4+ lymphocytes in two studies,^{28,29} also express the CD11b antigen in contrast to the situation in healthy individuals.²⁸ In this context, the reported tissue distribution of CD4+HNK1+ and CD8+HNK1+ lymphocytes may be particularly illuminating. Most HNK1+ lymphocytes in the germinal centers of immunologically activated lymph nodes are also CD4+.^{27,29-31} The CD8+HNK1+ lymphocytes are rare in tissues,²⁷ although they are predominant among the very few HNK1+ T cells in the bone marrow²¹ and must be considered as a predominantly bloodborne subset.²⁷ The increased numbers of HNK1+ lymphocytes coexpressing T cell markers in the blood of some patients with B cell malignancies, which represent a significant proportion of the cells in germinal centers, suggest that HNK1+ T cells have a regulatory role in B cell differentiation and expansion.^{32,33}

Our morphological, cytochemical, and ultrastructural studies of CD4+HNK1+ and CD8+HNK1+ T cells have revealed that those cells have a large cytoplasmatic to nuclear ratio, moderate numbers of electron-dense cytoplasmatic granules, and a moderately developed Golgi apparatus. More than 95% of those cells are acid phosphatase positive, but they are generally negative for nonspecific esterase activity. Thus, our CD4+HNK1+ T cells closely

resemble the peripheral blood CD4+ granular lymphocytes described by other researchers,^{31,34} of which a significant proportion expresses the HNK1 marker. The CD4+HNK1+ lymphocytes recovered from reactive tonsils show numerous electron-dense granules and abundant Golgi vesicles, suggesting that they are in a more active phase of granule formation than their peripheral blood counterparts.³¹

Morphological studies of CD8+CD11b+ T cells, of which 70% to 80% express HNK1, have revealed a scattered staining pattern for nonspecific esterase activity³⁵ and numerous electron-dense azurophilic granules and prominent Golgi apparatus.³⁶ In contrast to those studies, our CD8+HNK1+ T cells are generally negative for nonspecific esterase activity and lack the CD11b marker (data not shown). In addition, our ultrastructural studies suggest that our CD8+HNK1+ T cells are in a less active phase of granule formation. The CD8+HNK1+ lymphocytes that circulate in large numbers in the blood of long-term survivors after allogeneic BMT have only sparse granules or no granules at all.²⁶

Typically, T cells have a dotlike staining pattern for nonspecific esterase activity,³⁷ which changes to a granular pattern during the early phases of in vitro cell activation, whereas blast cells have lost their nonspecific esterase activity.^{38,39} The possibility that the CD4+HNK1+ and CD8+HNK1+ T cells in our study may represent activated T cells is contradicted by the observations that they express neither HLA-DR antigens nor interleukin 2 (IL 2) receptors (data not shown). Similarly, the CD8+HNK1+ T cells in long-term survivors of allogeneic BMT generally do not express HLA-DR antigens.²⁶ The lack of nonspecific esterase activity in CD4+HNK1+ and CD8+HNK1+ T cells may indicate that those cells have returned to a resting state after a previous phase of activation, eg, while combatting CMV infection.

Functional studies of peripheral blood CD4+ granular lymphocytes and tonsillar CD4+HNK1+ lymphocytes have shown neither helper nor suppressor activity in the pokeweed

mitogen (PWM) driven-Ig synthesis assay.^{31,40} In contrast, the peripheral blood CD8+CD11b+(HNK1+) lymphocytes of healthy individuals³⁶ and the CD8+(CD11b-) HNK1+ lymphocytes of long-term survivors of allogeneic BMT²⁶ show suppressor function in that assay. HNK1+ lymphocytes that also express T cell markers have low cytotoxic activities against tumor cell lines used as targets in NK assays.^{41,42} However, lectin-dependent cellular cytotoxicity (LDCC) can be induced even against NK-insensitive target cell lines, in peripheral blood HNK1+ T cells⁴³ and tonsillar CD4+HNK1+ lymphocytes.³¹ Consequently, the suppressive effects of HNK1+ T cells on PWM-induced Ig synthesis may be mediated by PWM-dependent cellular cytotoxicity against the PWM-responsive lymphocytes.⁴³

Our observation that the HNK1+ T cells that increase in number in association with CMV carrier status do not express NKH1 suggests that they do not represent the subset of T cells that mediates non-HLA restricted cytotoxicity, which has the CD3+NKH1+ phenotype.¹⁶ The suggestion that the HNK1+ T cells include in vivo primed antiviral cytotoxic T cells⁴³ is of interest in view of our observation that those cells increase in number proportionally to the levels of IgG-class CMV antibody titers. The identification of such in vivo primed anti-CMV cytotoxic T cells and the elucidation of their activation mechanisms may yield a new therapeutic option for immunocompromised patients in whom CMV reactivations can often be life-threatening. In conclusion, our results illustrate the unique position of CMV among the herpes viruses in interacting with the immune systems of its carriers, as reflected by its persistent effects on peripheral blood T lymphocyte subsets.

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Capacity of Human Serum to Depolymerize Actin Filaments

By Paul A. Janmey and Stuart E. Lind

Human blood depolymerizes filamentous (F-)actin. The interaction of actin filaments and monomers with human serum was studied by following the kinetics and extent of the depolymerization of pyrene-labeled F-actin and by analysis of serum proteins adhering to immobilized actin monomers. In physiologic Ca^{2+} concentrations, the depolymerization of F-actin proceeds in two stages: a rapid phase, attributed to direct severing of filaments by plasma gelsolin, and a slow phase attributed to the binding of actin monomers to vitamin D-binding protein (DBP). Without Ca^{2+} , only the slow phase is observed. Human serum can completely depolymerize 10 to 18 $\mu\text{mol/L}$ of actin, of which $\sim 5 \mu\text{mol/L}$ occurs rapidly. Depolymerization can be accounted for by the normal serum concentrations of

gelsolin and DBP. Fibrin(ogen) and fibronectin, which bind actin in vitro, do not contribute to the kinetics or extent of its depolymerization. Affinity chromatography and functional assays for the presence of gelsolin-actin complexes show that addition of G-actin to serum results in preferential formation of actin-DBP complexes, but that addition of F-actin to serum produces both gelsolin-actin complexes and DBP-actin complexes. The distinctive binding of actin monomers and polymers to these two serum proteins suggests a means by which their coordinated actions are maximized in vivo, from the standpoint of depolymerizing filaments and clearing monomers from the circulation.

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FILAMENTOUS actin (F-actin), a major constituent of all types of cells disassembles in the presence of human serum. Two plasma proteins have been described that can depolymerize F-actin by distinct mechanisms. Plasma gelsolin,¹⁻⁷ which is a variant of cytoplasmic gelsolin,⁸⁻⁹ severs actin filaments in the presence of Ca^{2+} and binds to their fast-growing ends. Vitamin D-binding protein (DBP) binds to monomeric actin (G-actin) with high affinity, causing depolymerization by perturbing the steady-state exchange of actin monomers from the filament ends. DBP does not bind to F-actin and is not affected by Ca^{2+} .¹⁰⁻¹⁴ Other plasma proteins (fibrin,¹⁵⁻¹⁷ fibronectin,^{18,19} IgG,²⁰ and Clq ²¹) also bind actin but their effects on actin polymerization have not been fully determined.

The physiologic significance of the binding of these plasma constituents to actin has not been firmly established. Actin is, however, the most abundant protein in most cells²² and has been detected in blood serum under some circumstances,^{6,23-25} possibly as the result of tissue injury or normal cell turnover. The presence of actin-depolymerizing activity in blood suggests that actin filaments in the extracellular space may have deleterious effects. F-actin may raise blood viscosity in the microvasculature or interfere with normal fibrin clot formation.¹⁷

Actin depolymerization may also be facilitated by factors other than proteins. Millimolar concentrations of Ca^{2+} and low levels of ATP, both of which decrease actin polymer stability in vitro,^{26,27} may also contribute to the depolymeriz-

ing activity of blood. Previous studies suggested that the functions of purified gelsolin and DBP are maximally expressed at the ionic conditions present in serum,^{28,29} but whether they account fully for the activity of whole serum is not known.

Although the amount of actin that is depolymerized by blood has been estimated,³ there has been no systematic quantitative study of the capacity of human blood serum to depolymerize actin. We report such measurements and account for the depolymerizing activity in terms of the concentrations and activities of the proteins plasma gelsolin and DBP.

MATERIALS AND METHODS

Materials. DNAase I was purchased from Sigma Chemical (St Louis). CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) was coupled to DNAase I (1 mg/mL of hydrated beads) according to the manufacturer's instructions. *N*-(1-pyrenyl)-iodoacetamide was obtained from Molecular Probes (Eugene, OR).

Serum preparation. Serum was prepared by allowing blood to clot in a glass tube for 2 hours at 37 °C and then centrifuging it for 20 minutes at 3,000 g. Plasma was prepared from ACD-anticoagulated blood supplied by the blood bank and was centrifuged twice at 3,000 g for 20 minutes in plastic containers to remove cellular elements.

Protein purification. Gelsolin was prepared from plasma by monoclonal anti-gelsolin antibody affinity chromatography by the method of Chaponnier and colleagues.³⁰ DBP was prepared from plasma by the method of Haddad and associates³¹ following passage of the plasma over a column of Sepharose 4B coupled to anti-gelsolin antibody.³⁰ Actin was prepared from rabbit skeletal muscle³² and labeled with *N*-(1-pyrenyl)-iodoacetamide as described elsewhere.³³ Pyrene-labeled actin was stored frozen in liquid nitrogen as monomeric (G-) actin in 2 mmol/L of Tris(hydroxymethyl)aminomethane (Tris), 0.2 mmol/L of ATP, 0.2 mmol/L of CaCl_2 , 0.2 mmol/L of 2-mercapto-ethanol, pH 7.8. It was diluted 1 day before measurements were made and mixed with various amounts of unlabeled actin.

Functional assays for serum gelsolin concentration. The total gelsolin concentration (free or bound to actin) of serum or plasma samples was determined from their ability to accelerate actin assembly.^{34,35} The concentration of free gelsolin (ie, not bound to actin monomers) was determined by measuring the filament-severing ability of the serum. The assay is based on the fact that when pyrene-labeled actin filaments are severed by gelsolin and then diluted below the critical concentration of the slow-exchanging (-)

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filament end (0.5 to 1.2 $\mu\text{mol/L}$), the rate at which such filaments depolymerize is proportional to the number of (—) ends,^{34,36} which is equal to the number of cuts made by gelsolin. Previous experiments have shown that preincubation of purified gelsolin with actin inhibits its severing activity such that nearly all filament-severing activity is lost at a molar ratio of actin to gelsolin of 2:1.³⁷

Measurement of actin depolymerization by serum. Depolymerization of F-actin by serum samples was measured by first adding pyrene-labeled actin filaments (polymerized by addition of 2 mmol/L of MgCl_2 and 150 mmol/L of KCl) to the sample in a cylindrical glass light-scattering tube (6-mm diameter, 200- μL sample volume), gently mixing the solution by drawing it up in a Pasteur pipette, and then placing the scattering tube in a fluorometer (Perkin Elmer LS-5, Oak Brook, IL). The time between mixing and the first fluorescence measurement was 5 to 10 seconds. The actin content of some samples was varied by altering the concentration of unlabeled actin, keeping the pyrene-actin concentration constant prior to polymerization with added salts. Pyrene-labeled actin copolymerizes randomly with unlabeled monomers under these conditions.³⁸ Some serum samples were dialyzed into buffers containing 50 mmol/L of Tris and 150 mmol/L of NaCl (TBS) to assess the possible effects of dialyzable factors, especially Ca^{2+} . Some serum samples were depleted of gelsolin by passing 4 mL of serum over a 1.5-mL column of Sepharose-coupled anti-gelsolin antibody equilibrated with TBS plus 3 mmol/L CaCl_2 (TBS- Ca^{2+}). Control whole serum was diluted to an equal total protein concentration.

Affinity chromatographic assay for actin binding. Varying amounts of G-actin were added to serum. After a 10-minute incubation at 23 °C, the serum-actin mixture was added to 40 μL of DNAase I immobilized on Sepharose 4B beads. The mixture was incubated with DNAase beads for 2 hours at 4 °C with continuous end-over-end tumbling. The DNAase beads were sedimented by centrifugation at 18,000 g for 1 minute and washed once in TBS- Ca^{2+} with 0.75% Triton X-100 (Sigma, St Louis) and twice with TBS- Ca^{2+} . Proteins adhering to the DNAase beads were eluted with the gel sample buffer of Laemmli,³⁹ analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their mol wt was compared to known standards. The amounts of DBP and gelsolin bound to actin were quantified by scanning Coomassie blue-stained bands with a laser scanning device and multiplying the relative areas by the ratios of mol wt of actin (42 kd), DBP (56, kd), and gelsolin (93 kd).

RESULTS

Kinetics of actin depolymerization by serum with and without Ca^{2+} . The depolymerization of F-actin by serum depends on the free Ca^{2+} concentration, as has been reported previously by measurements using other assays.^{1,40} As shown in Fig 1, depolymerization of F-actin added to whole serum proceeded rapidly. Removal of Ca^{2+} severely retarded the initial rate of depolymerization and its final extent. Addition of Ca^{2+} to depleted samples restored the rate to that in whole serum. The depolymerization in Ca^{2+} proceeds in two stages: a rapid initial phase that is completed in several seconds followed by a slower phase that occurs over a period of many minutes. Without Ca^{2+} , the initial rapid phase of depolymerization does not occur. Instead, a slow steady decrease in F-actin fluorescence occurs, leading, after several hours, to partial depolymerization.

Capacity of serum to depolymerize actin. Various amounts of actin were added to serum (in the presence of Ca^{2+}) to determine the filament-depolymerizing capacity of serum. Depolymerizing activity was resolved into slow and

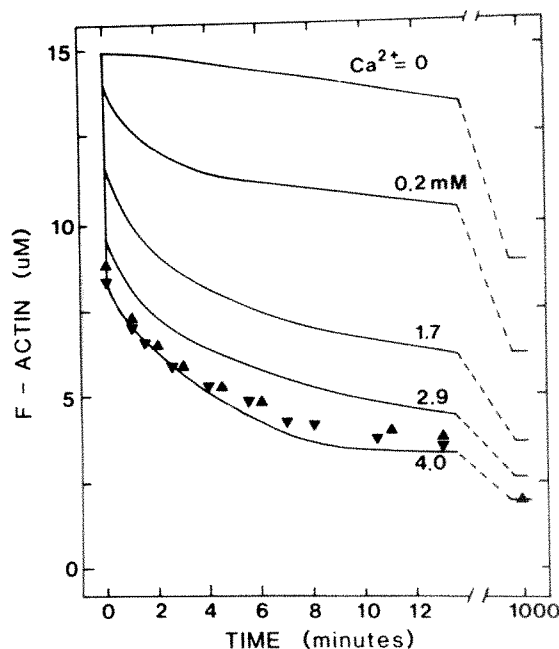


Fig 1. Kinetics of depolymerization of actin filaments in serum in the presence and absence of Ca^{2+} . Pyrene-labeled F-actin (15 $\mu\text{mol/L}$ final concentration) was added to serum (87% vol/vol final concentration), and the depolymerization of actin was measured by the decrease in fluorescence. The time-course of depolymerization in the presence of whole serum ($\blacktriangle$, ∇ , two separate experiments) is compared with that in the presence of serum dialyzed against 50 mmol/L of Tris, 150 mmol/L of NaCl (TBS), to which Ca^{2+} was added back to the concentrations indicated (—)

rapid phases by monitoring the amount of filamentous actin present 30 seconds and 7 hours after addition of serum (Fig 2). The two phases were saturated at different actin concentrations. The slow phase of depolymerizing activity was saturated at three to four times the actin concentration

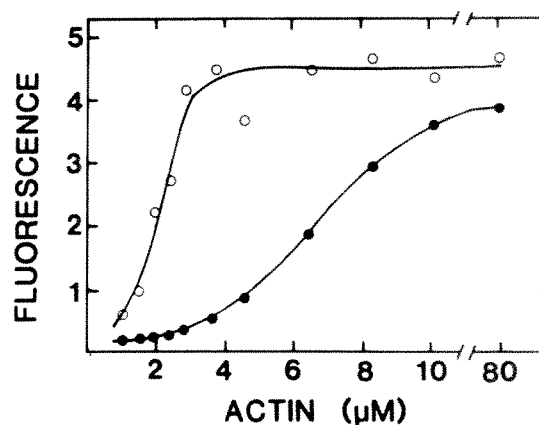


Fig 2. Relative extents of fast and slow depolymerization of various concentrations of F-actin added to serum. Pyrene-labeled actin (1 $\mu\text{mol/L}$ final concentration) was copolymerized with various concentrations of unlabeled actin and then mixed with serum and buffer 50 mmol/L of Tris, 150 mmol/L of NaCl (TBS). The fluorescence of the pyrene-actin was measured 30 seconds ($\circ$) and 7 hours ($\bullet$) after addition of serum. The final mixtures contained 46% by volume of serum and the gelsolin concentration measured by nucleation assays was 0.74 $\mu\text{mol/L}$.

required to saturate the rapid phase. The fraction of total depolymerization that occurs during the initial phase is a function of the amount of F-actin added to the serum. Measurement of the gelsolin concentration of the specimen showed that the rapid phase was saturated at a ratio of actin to gelsolin of 2:1. The relative extents of the fast and slow depolymerization phases as a function of the added actin concentration are shown in Fig 2, which shows the fluorescence of pyrene-labeled actin added to a serum solution containing 0.69 $\mu\text{mol/L}$ of gelsolin measured 30 seconds and 7 hours after the addition of F-actin samples containing a constant amount of pyrene-labeled actin copolymerized with different amounts of unlabeled actin. When the total actin concentration was $<1.7 \mu\text{mol/L}$, nearly all the fluorescence was eliminated in 30 seconds, suggesting that when a molar ratio of $<2:1$ of actin to gelsolin exists, depolymerization proceeds rapidly. When 4 $\mu\text{mol/L}$ of total actin was added, nearly 90% of the initial fluorescence was observed after 30 seconds, but the fluorescence decrease was nearly total after 7 hours, suggesting that the actin eventually depolymerized by a different mechanism. When the final actin concentration was $>10 \mu\text{mol/L}$, the fluorescence was only partially decreased even after 7 hours, suggesting that most of the actin remained in polymeric form. Both the fast and slow phases of depolymerization are saturable. The rapid phase was saturated when the ratio of actin to gelsolin was $\sim 2:1$, but saturation of the slow phase of depolymerization was observed at approximately threefold higher actin concentrations.

Effect of low concentrations of F-actin on the filament-severing and polymerization-accelerating activities of serum. The severing activities of serum samples (total gelsolin concentration 0.7 $\mu\text{mol/L}$) containing different concentrations of unlabeled F-actin are shown in Fig 3. The free gelsolin concentration (severing activity of the serum) decreased in direct proportion to the amount of unlabeled F-actin added during the preincubation step, with nearly total loss of severing activity at a concentration of 1.5 μmol of actin, a molar ratio of nearly 2:1 with respect to gelsolin. Although such a serum sample could not sever actin filaments, it had depolymerizing activity since, as shown in Fig 2, nearly 5 $\mu\text{mol/L}$ of actin was totally depolymerized by serum samples after several hours. This result suggests that the slow phase of depolymerization is due to a component of serum that does not sever actin filaments. In contrast to their effects on the filament-severing activity of serum, the presence of these concentrations of F-actin had no effect on the ability of serum to nucleate actin assembly from monomers, as shown in Fig 3. This result is characteristic of the effects of actin on purified gelsolin.³⁷

Different effects of G-actin and F-actin on the depolymerizing properties of serum and mixtures of gelsolin and DBP. If G-actin rather than F-actin was added to serum, the severing activity of the serum was not diminished, as shown in Fig 4. The initial rapid phase of depolymerization was eliminated by prior incubation of serum with F-actin. In contrast, when an equivalent amount of G-actin was added, the rapid phase of depolymerization was nearly the same as that which occurred with control serum.

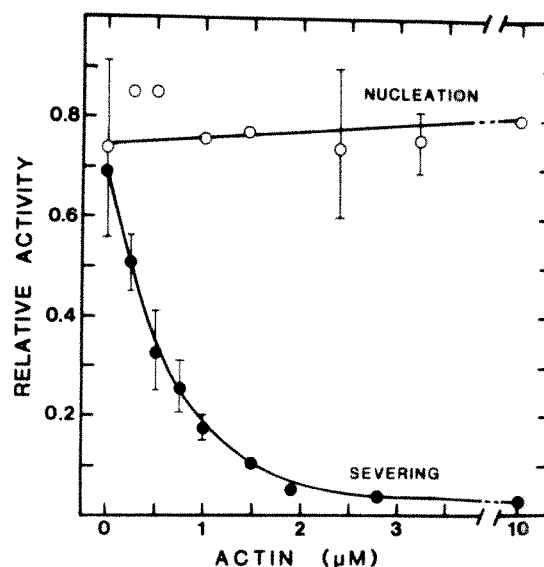


Fig 3. Differential effects of addition of F-actin to serum on the ability of serum to sever actin filaments or to nucleate actin polymerization. Serum (100 μL) was added to 120 μL of various concentrations of actin in 50 mmol/L of Tris, 150 mmol/L of NaCl (TBS). After 2 hours of incubation at room temperature, aliquots of the samples were used to measure their effect on the depolymerization of pyrene-labeled F-actin or on the rate of polymerization of pyrene-labeled G-actin. For determination of the severing ability of the serum-actin mixtures, 20 μL of the serum-actin samples was added to 15 μL of pyrene-labeled F-actin (11 $\mu\text{mol/L}$), and the mixture was diluted with buffer B (buffer A with 2 mmol/L of MgCl_2 and 150 mmol/L of KCl) to a final pyrene-actin concentration of 0.15 $\mu\text{mol/L}$. For the nucleation reaction, 10 μL of sample was added to 2.8 $\mu\text{mol/L}$ of pyrene-labeled G-actin in 200 μL of buffer B.

Effects of depleting gelsolin from serum on actin depolymerization. Because gelsolin is believed to be necessary for the rapid phase of depolymerization through its direct severing of filaments, depletion of gelsolin from serum would be expected to eliminate the rapid phase of F-actin depolymerization. Such a loss of the rapid phase was observed, as shown in Fig 5. Addition of purified gelsolin to the gelsolin-depleted serum restored the depolymerization rate to that observed in the presence of whole serum.

Both rapid and slow phases of depolymerization can be observed in mixtures of purified gelsolin and DBP. The time-course of depolymerization of actin filaments added to a mixture of purified gelsolin and DBP, shown in Fig 6, is similar to that of F-actin added to serum, shown in Fig 5. The rate and extent of depolymerization are greater in the presence of both gelsolin and DBP than in DBP alone (Fig 6). Depolymerization in the presence of gelsolin alone (Fig 6) accounts for the rapid phase of depolymerization but does not account for the final extent of depolymerization observed in the presence of both gelsolin and DBP. Adding unlabeled F-actin to gelsolin-DBP mixtures prior to addition of pyrene-F-actin eliminates the rapid depolymerization consistent with F-actin binding preferentially to gelsolin. Adding an equal amount of G-actin does not affect the rapid phase but decreases the final extent of depolymerization suggesting binding primarily to DBP (Fig 6). The depoly-

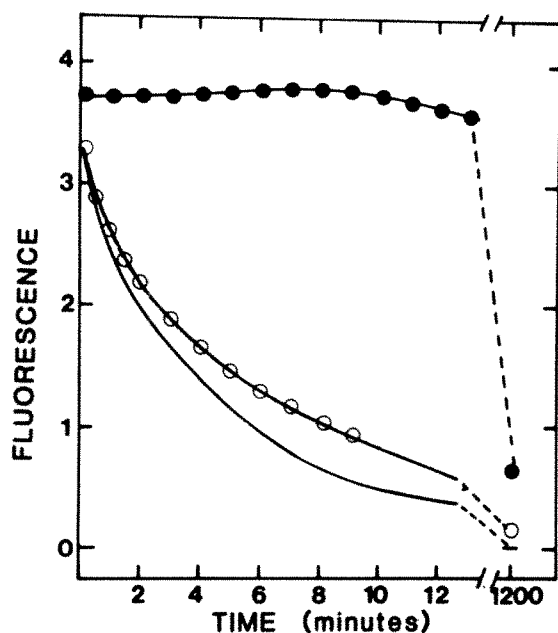


Fig 4. Severing and depolymerization of actin filaments by serum following incubation with G-actin or F-actin. Human serum (15 μ L) was incubated with 5 μ L of 12 μ mol/L of G-actin (○) or F-actin (●) for 1 minute. The mixture was added to 500 μ L of buffer B; 15 μ L of 12 μ mol/L of pyrene-labeled F-actin was then added, and the depolymerization of the labeled actin filaments was monitored by the decrease in sample fluorescence. The fluorescence of pyrene-labeled G-actin was subtracted from all measurements.

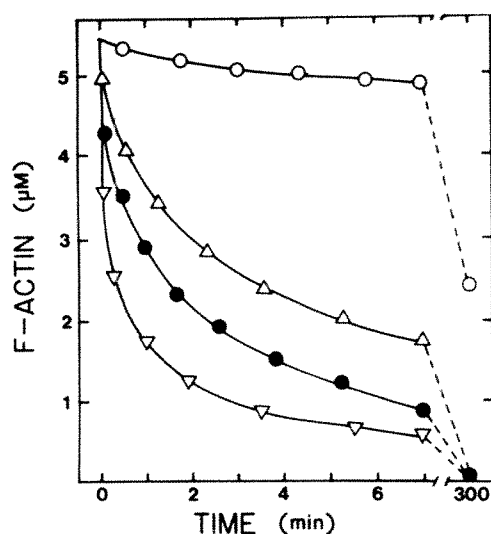


Fig 5. Effect of depletion of gelsolin from serum on its ability to depolymerize F-actin. The time-course of depolymerization of 5.5 μ mol/L of pyrene-labeled F-actin in whole serum (45% vol/vol final concentration, ●) is compared with that of homologous serum that had been depleted of gelsolin (○). Addition of purified plasma gelsolin to the depleted serum (0.4 μ mol/L, Δ; 0.6 μ mol/L, ▽) restored the depolymerization rate to near that of whole serum.

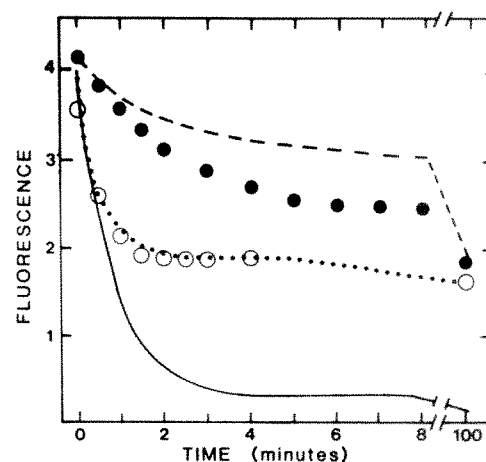


Fig 6. Depolymerization of F-actin in the presence of purified gelsolin and vitamin D-binding protein (DBP). Pyrene-labeled F-actin (3.6 μ mol/L final concentration) was added to solutions in buffer B containing 0.52 μ mol/L of gelsolin (···), 1.2 μ mol/L of DBP (—) or both 0.52 μ mol/L of gelsolin and 1.2 μ mol/L of DBP (— · —). In separate experiments, the solution containing both gelsolin and DBP was incubated for 2 minutes with either 1 μ mol/L of G-actin (○) or 1 μ mol/L of F-actin (●) prior to addition of pyrene-labeled F-actin.

merization of F-actin by the purified proteins was not affected by the addition of physiologic concentrations of fibrinogen or fibronectin, (data not shown).

Preferential binding of G-actin to DBP in the presence of free gelsolin. G-actin was added to serum and then isolated together with bound proteins from the mixture with DNAase-Sepharose. (DNAase I has a high affinity for G-actin⁴² and forms ternary complexes containing DNAase-actin-DBP⁴³ or DNAase-actin-gelsolin.⁴⁴) As shown in Fig 7B, polypeptides of mol wt 56,000, 66,000, and 93,000 were observed when G-actin was added to serum. The 93,000 polypeptide comigrates with gelsolin, immunoprecipitated from the same sample by immobilized monoclonal anti-gelsolin antibodies (lane A). The 56,000 polypeptide is presumed to be DBP. These two polypeptides are bound to actin, since they are not precipitated by DNAase beads alone. The 66,000 polypeptide is presumed to be albumin. Its binding appears to be nonspecific, because this band was also observed for samples prepared from serum without added G-actin, and the amount of this polypeptide did not generally correlate with the amount of added actin. The amounts of DBP and gelsolin that coprecipitated with various concentrations of actin are shown in Fig 7A. For these samples, which contained 50% serum by volume, an equimolar amount of DBP was bound to actin at actin concentrations up to 4 μ mol/L and binding appeared to be saturated at 6 μ mol/L of DBP. Almost no gelsolin was apparent when <4 μ mol/L G-actin was added to the samples; thereafter the amount of gelsolin increased. This finding confirms that G-actin binds preferentially to DBP, and when this binding is complete, actin monomers bind also to gelsolin.

DISCUSSION

Undiluted serum can depolymerize 10 to 18 μ mol/L of F-actin. Of this total, ~5 μ mol/L is depolymerized immedi-

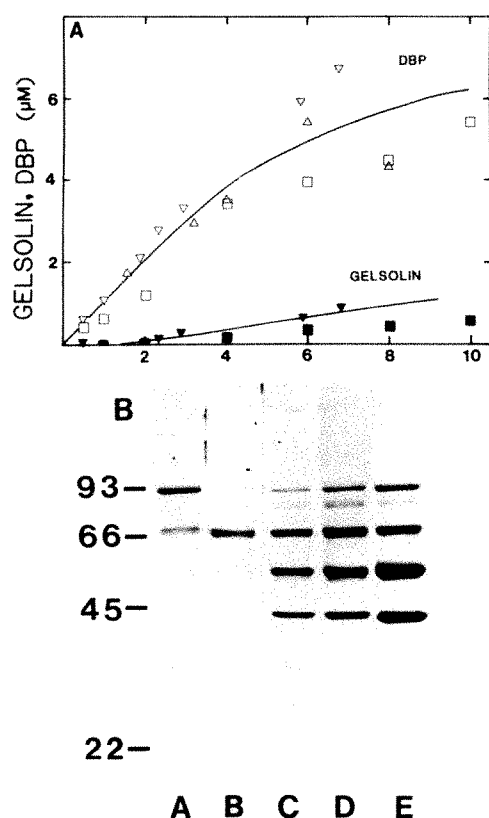


Fig 7. Isolation of G-actin-containing complexes from serum. (A) Increasing amounts of G-actin were added to mixtures of 100 μ L of serum and 100 μ mol/L of 50 mmol/L of Tris, 150 mmol/L of NaCl (TBS). Actin-containing complexes were isolated with DNAase-Sepharose beads. After washing, the beads were incubated with gel sample buffer and loaded on 5% to 15% minigels, and the gels were stained with Coomassie blue. The amount of gelsolin (closed symbols) and vitamin D-binding protein (DBP) (open symbols) coprecipitating with different amounts of actin were determined from densitometry of Coomassie-stained bands. Different symbols represent separate experiments. (B) Representative lanes are shown for proteins adhering to DNAase beads added to serum containing 0.5 (b), 1.5 (c), 3.0 (d), or 7 (e) μ mol/L of G-actin. Lane A: Serum proteins bound to immobilized antigelsolin antibodies.

ately; this rapid depolymerization is due primarily to rapid severing of filaments by gelsolin, which has two actin-binding sites and is present in human serum at a concentration of 1.2 to 3.5 μ mol/L.⁴⁵ An additional 6 to 12 μ mol/L of actin is depolymerized in a slow, Ca^{2+} -insensitive manner. These properties suggest that DBP, present in serum at concentrations of 6 to 10 μ mol/L,¹² is responsible for the slow phase of depolymerization. The initial depolymerization is also dependent in part on the Ca^{2+} level of serum, which is much higher than that of cytoplasm. Although gelsolin is Ca^{2+} sensitive, it is maximally active when Ca^{2+} is >10 μ mol/L, whereas differences with serum are observed up to 2 mmol/L. The Ca^{2+} effect is not a direct effect on fluorescence, since no rapid fluorescence change is observed in the absence of gelsolin. The apparent sensitivity to millimolar Ca^{2+} concentrations may reflect destabilization of actin filaments²⁶ rather than augmentation of gelsolin function.

Because the depolymerization of actin filaments by serum is reproduced by solutions containing physiologic concentrations of purified gelsolin and DBP, these two proteins account for most of the actin-depolymerizing action of serum, with a minor additional effect contributed by calcium ions.

Serum gelsolin and DBP function in a coordinated manner to maximize their actin-depolymerizing properties. If serum contained only gelsolin, it would lose its filament-severing capacity as soon as actin appeared extracellularly, even if only actin monomers were released. The presence of DBP, to which actin monomers bind preferentially, ensures that gelsolin will not become saturated with actin monomers, but will be able to sever actin filaments. Thus, DBP prevents the loss of the rapid filament-severing activity of serum due to release of G-actin. The preservation of gelsolin's filament-severing capacity complements DBP's role as the major actin-clearing protein of blood.²⁹

The significance of serum actin-depolymerizing proteins has become more apparent in the light of recent evidence of actin in the extracellular space.^{6,23-25} The difficulty of documenting the presence of actin in the blood may be due to the efficiency of the proteins that sequester and clear it (gelsolin and DBP²⁹). Alternatively, its affinity for fibrin^{15,17} may result in its being inadvertently discarded with the fibrin clot when serum is prepared.

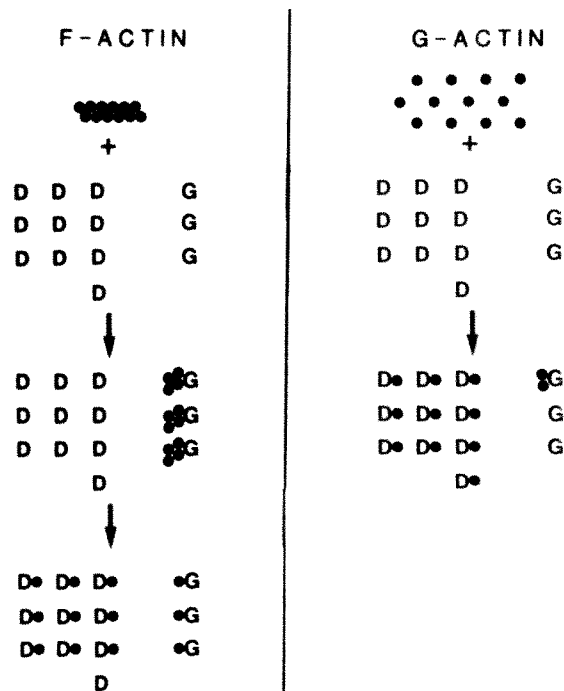


Fig 8. Schematic of complexes formed following addition of G-actin or F-actin to gelsolin and vitamin D-binding protein (DBP). Diagrams indicate the different mechanisms by which complexes are formed when actin filaments or monomers are added to a solution containing gelsolin and DBP at their appropriate molar ratio in human blood. When F-actin is added, complexes form first with gelsolin and secondarily with DBP. If an equivalent amount of G-actin is added, complexes form preferentially with DBP. Most of the gelsolin remains free and capable of severing actin filaments.

The combined effects of gelsolin and DBP (at their physiologic molar ratios) on G-actin and F-actin are summarized in Fig 8. When F-actin is added to serum, it binds first to gelsolin to form filament fragments that have a gelsolin cap on the (+)-end. DBP can then remove actin monomers from these complexes, forming 1:1 actin-DBP complexes, until all the DBP is complexed. If less than $\sim 15 \mu\text{mol/L}$ of actin is added (ie, the amount of F-actin that can be totally depolymerized), 1:1 actin-gelsolin complexes will remain as the DBP-actin complexes form. If instead, the same amount

of G-actin is added, the actin monomers bind first to DBP. When all DBP has formed 1:1 complexes with actin, the remaining actin monomers bind to gelsolin. Because the binding of actin to gelsolin is cooperative,^{37,41,46} the serum will contain a mixture of 2:1 actin-gelsolin complexes and free gelsolin, but not 1:1 actin-gelsolin complexes.

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Hemophilia A: Carrier Detection and Prenatal Diagnosis by DNA Analysis

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In this study, we used DNA polymorphisms for carrier detection and prenatal diagnosis of hemophilia A in a large group of Italian families. The restriction fragment length polymorphisms (RFLPs) investigated were the intragenic polymorphic *Bcl*I site within the factor VIII gene; the extragenic multiallelic *Taq*I system at the *St14* locus; and the extragenic *Bgl*II site at the *DX13* locus. The factor VIII probe was informative in 30%, *St14* in 82%, and *DX13* in 60% of obligate carriers. The combination of factor VIII-*Bcl*I and *St14*-*Taq*I showed that 91% of obligate carriers were heterozygotes for one or both; with all three probes, only 4% of obligate carriers were noninformative. In families clearly segregating for hemophilia A, RFLP analysis allowed us to define the carrier status for the hemophilia A gene in all 27 women tested. RFLP analysis allowed us to exclude

the carrier status in 39 of 45 female relatives of sporadic patients. The combination of RFLP analysis and biological assay of factor VIII allowed us to identify a de novo mutation in the maternal grandfather in 7 of 12 of the families with sporadic cases, for which members of three generations were available for study. Nine of 10 couples requesting prenatal diagnosis provided informative RFLP DNA pattern. Carrier status was excluded in two women, two fetuses were shown to be female, and prenatal diagnosis was carried out in five pregnancies by DNA analysis. Prenatal testing was successful in three instances and failed in two because a sufficient amount of chorionic villous DNA was not obtained for the analysis.

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HEMOPHILIA A is the most common human bleeding disorder, affecting ~1 male in 10,000. It results from a deficiency or abnormality of clotting factor VIII-C and is inherited as a X-linked trait.¹ The locus for factor VIII has been assigned to band q28 of chromosome X.² Until a few years ago, carrier detection was based on estimation of the ratio of factor VIII coagulant activity (FVIII-C) to von Willebrand factor antigen (vWF:Ag), which is accurate in 80% of cases.³⁻⁶ Prenatal diagnosis has been carried out by conventional analysis of fetal blood obtained at 18 to 20 weeks of gestation by fetoscopy,^{7,8} a procedure associated with a high risk of fetal mortality. Recently, the factor VIII gene was cloned⁹⁻¹¹ and three¹² DNA polymorphisms within the factor VIII-C were detected.¹²⁻¹⁴ In addition, random X chromosome-specific DNA probes, known *DX13*¹⁵ and *St14*,¹⁶ closely linked to the factor VIII gene, detected several restriction fragment length polymorphisms (RFLPs). These discoveries have been applied to carrier detection^{12,17-20} and prenatal diagnosis of hemophilia A.²¹⁻²² The intragenic polymorphisms were informative only in a limited number of the families investigated, whereas the *DX13* and, less frequently, the *St14* probes showed recombination with the factor VIII locus.^{17,19,22,23}

In this article, we describe the use of a factor VIII cDNA probe in combination with X chromosome-specific random probes, *DX13* and *St14*, for carrier detection and prenatal diagnosis of hemophilia A in a large series of families of Italian descent.

MATERIALS AND METHODS

Subjects. After informed consent was obtained, blood samples were collected from members of 61 families followed at the hemophilia center of Gaslini Institute in Genoa, the Pediatric Clinic in Palermo, the hematology department of Carreggi Hospital in Florence, and the 2nd medical clinic and the hemophilia center of Pellegrini Hospital in Naples.

Coagulation and immunologic assays. Factor VIII coagulant activity (VIII-C) was measured by a one-stage method²⁴; vWF:Ag was determined by a modification of Laurell's technique.²⁵

DNA analysis. Venous blood samples were collected into citrate, and the DNA was extracted from WBCs by standard

techniques.²⁶ Chorionic villous samples were obtained by the trans-cervical route under ultrasound guidance using a malleable silver cannula.²⁷ The chorionic villous samples were checked microscopically to exclude contamination with maternal decidua before being dissolved in 10 mmol/L of Tris-HCl (hydroxymethylaminomethane hydrochloride) pH 7.5, 1 mmol/L of EDTA, 2% sodium dodecyl sulfate (SDS), 8 mol/L of urea, and 0.3 mol/L of NaCl. This was followed by extraction with phenol and then chloroform. The DNA was then precipitated with ethanol and reconstituted in TE buffer (1 mmol/L of Tris-HCl pH 7.5, 1 mmol/L of EDTA); 10 µg of DNA was digested with the restriction endonucleases *Bcl*I, *Bgl*II, or *Taq*I (Amersham, UK), under conditions recommended by the manufacturers. Resulting fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose filters by Southern blotting.²⁸ Bound DNA was hybridized with probes radiolabeled with ³²P by nick-translation.²⁹ Hybridization was performed overnight at 42 °C in a solution containing 50% formamide, 50 mmol/L of Hepes, pH 8.0, 5 × Denhardt's [1 × = 0.2% bovine serum albumin (BSA), 0.2% polyvinyl-pyrrolidone, 0.2% Ficoll 400], 6 × 1 × = 0.15 mol/L of sodium chloride and 0.015 mol/L of sodium citrate (SSC),

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200 µg of salmon sperm DNA, 10% dextran sulfate and 4×10^6 cpm/mL of heat-denatured probe. After hybridization, excess probe was washed off in $2 \times$ SSC for 30 minutes at room temperature, followed by $1\frac{1}{2}$ hours at 50°C in $0.1 \times$ SSC and 0.1% sodium dodecyl sulfate (SDS). DNA bands that hybridized to the ^{32}P -labeled probe were detected by autoradiography with x-ray film (X-Omat, AR; Kodak) on intensifying screens (Cronex, Du Pont, UK).

DNA probes used were the 1.3-kilobase (kb) fragment of factor VIII cDNA covering exons 14 through 19 (kindly provided by Chiron Corporation, Emeryville, CA)¹¹; DX13, a cloned sequence localized to band Xq28 by somatic cell hybrids³⁰ and by in situ hybridization³¹ (kindly provided by Dr K. E. Davies); and St14, a 3-kb DNA fragment originating from the q26-qter region of the X chromosome¹⁶ (kindly provided by Dr J. L. Mandel).

RESULTS

Frequency of intragenic and extragenic polymorphisms. We analyzed 61 hemophiliac DNA samples from unrelated families and 350 DNA samples from their relatives for the presence of a common *Bcl*I polymorphism within the factor VIII gene detected with a probe for exons 14 through 19, as well as for two common X chromosome RFLPs, which are detected by anonymous DNA sequences referred to as DX13 and St14, closely linked to the factor VIII gene. Typical Southern blotting analysis and RFLPs are shown in Fig 1. The *Bcl*I polymorphism 3' to the exon 18 of factor VIII gene yields a 879-base pair (bp) fragment when the site is present and 1,185 bp when the site is absent. The St14 probe hybridized to human DNA digested with *Taq*I reveals polymorphic patterns with at least ten allelic fragments in addition to fragments of constant size.^{16,18} The DX13 probe hybridized to human DNA digested with *Bgl*II detects a RFLP with allelic DNA fragments of 5.8 and 2.8 kb in length. The frequencies of the allelic DNA fragments at the three polymorphic systems investigated are summarized in Table 1. Twenty-seven obligate carriers from families with at least two affected children in separate sibships were also investigated to define the proportion who were heterozygotes for at least one polymorphism and in whom the linkage phase between the allelic DNA fragments and the hemophilia A locus was detectable (informative polymorphism). The factor VIII probe was informative in 9 of 27 (30%), St14 was informative in 19 of 23 (82%), and DX13 was informative in 14 of 23 (60%). Some subjects showed heterozygosity for more than one RFLP. When the factor VIII and St 14 probe were combined, 22 of 24 (91%) obligate

Table 1. Frequencies of Allelic DNA Fragments at Three Polymorphic Systems Intra (*Bcl*I/FVIII) and Extra (*Taq*I/St14, *Bgl*II/DX13) the Factor VIII Gene

Restriction Enzyme/Probe	<i>Bcl</i> I/FVIII	<i>Taq</i> I/St14	<i>Bgl</i> II/DX13
A.			
No. of X chromosomes studied	153	173	156
alleles in kb/%	1.1/35 0.8/65	6.6/0.6 5.3/4.6 4.8/15 4.5/31.8 4.0/15 3.9/18 3.4/14.5	5.8/43 2.8/57
B.			
Total no. of unrelated females studied	59	65	48
No. of heterozygotes for RFLPs/%	21/35	47/73	31/48
C.			
Total no. of unrelated obligate carriers studied	27	23	23
No. of heterozygotes for RFLPs/%	9/30	19/82	14/60

carriers were heterozygous for one or both polymorphisms. When the three probes were combined, 23 of 24 (96%) carriers were informative. Eighteen women, homozygous at the *Bcl*I site, were also analyzed for the intragenic *Bgl*II polymorphism.¹³ In none of them was this polymorphic site informative (data not shown).

Linkage of factor VIII to other markers. The linkage of St14 and DX13 locus to the *Bcl*I polymorphism revealed by the factor VIII probe was investigated in 20 and 19 families, respectively (Table 2). The number of meiotic events analyzed was 36 for the St14 locus and 31 for DX13. No recombination was observed between the St14 and factor VIII loci. The lod score for linkage of factor VIII to St14 is 4.8 at a recombination fraction of 0.0. This indicates that factor VIII gene is <6.4 recombination units with 95% confidence limits from the St14 locus. One recombinant was observed between DX13 and factor VIII/St14 loci (Fig 2). The lod score for linkage of factor VIII to DX13 is 2.1 at a recombination fraction of 0.045, indicating that factor VIII gene is <17.6 recombination units, with 95% confidence limits from DX13 locus.

Carrier detection. Carrier detection (Table 3) was carried out in 27 families with at least two hemophiliac males in

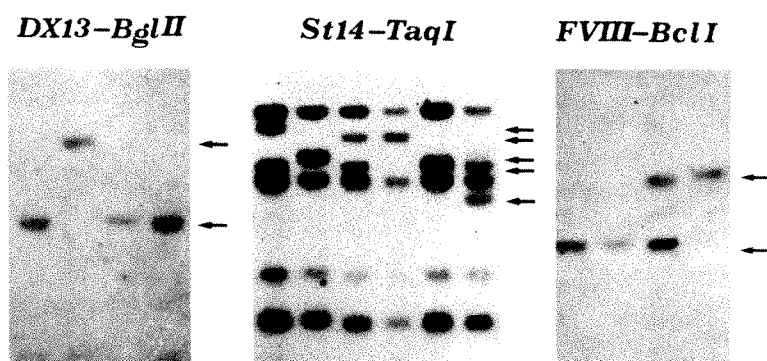


Fig 1. Autoradiogram of leukocyte DNA hybridized to DX13 (left), St14 (middle), and FVIII probes (right) following digestion with *Bgl*II, *Taq*I, and *Bcl*I, respectively. Arrows: restriction fragment length polymorphisms (RFLPs) at each locus were investigated and were: 5.8 and 2.8 kb for DX13; 4.8, 4.5, 4.0, 3.9, 3.4 kb for St14; 0.8 and 1.1 kb for FVIII.

Table 2. Linkage of Factor VIII Locus to Other Markers

Pairwise Cross	No. of Recombinants Total Meiosis	Recombination Fraction	Lod Score
FVIII × St14	0/36	0	4.2
FVIII × DX13	1/31	0.045	2.1

different sibships (group I) and in 34 with one or more hemophiliacs in only one sibship (group II). In the first group in which inheritance was clearly established, we investigated 29 women at risk of being carriers on the basis of genetic criteria. The *Bcl*I site revealed by the factor VIII probe was informative in 30% of cases, the *Taq*I system revealed by the St14 probe in 82%, and *Bgl*II revealed by the DX13 probe in 60%. RFLP analysis allowed classification of 11 (33%) women as carriers and 18 (67%) as non-carriers of the hemophilia A gene. None of the possible carriers tested escaped identification. In obligate carriers, the ratio of factor VIII-C/vWF:Ag was in the carrier range in 16 of 19 females (84%) investigated. Definition of the carrier status by RFLP analysis agreed with the results of the ratio of conventional factor VIII-C/vWF: Ag in 12 of 13 individuals in whom both assays were carried out.

In the sporadic cases (group II), the factor VIII probe was informative in 16 of 34 families (47%), the St14 was informative probe in 20 of 30 (66%), and the DX13 probe was informative in 15 of 30 (50%). The combination of the St14 and factor VIII probes was informative in 29 of 34 families (86%). By adding the analysis of the DX13 probe, the number of informative families was increased to 32 of 34 (94%). In only two of 34 families (6%) no informative RFLPs were found. RFLP analysis allowed exclusion of the carrier status in 39 of 45 (86%) female relatives of sporadic cases. In those families in which the mother was classified as a carrier on the basis of the conventional factor VIII assay, RFLP analysis allowed us to establish the carrier status in all four investigated females at risk. The biologic assay and

Table 3. Hemophilia A Carrier Detection by RFLP Analysis

Factor	Group I	Group II
	Families With Inherited Cases	Families With Sporadic Cases
No. of families	27	34
No. of possible carriers investigated	29	45
Carriers' status excluded (%)	67	86
Carriers' status identified (%)	33	14
Defined by (%)		
<i>Bcl</i> I/FVIII	30	47
<i>Taq</i> I/St14	82	50
<i>Bgl</i> II/DX13	60	41
<i>Bcl</i> I/FVIII- <i>Taq</i> I/St14	91	86

RFLP analysis were concordant in 17 of 25 carriers investigated. In 7 of 12 families, in which members of three generations were available for study, the combination of the biologic assay with RFLP analysis revealed a de novo occurrence of hemophilia A mutation in the maternal grandfather. An example of this type of de novo mutation is shown in Fig 3.

Prenatal diagnosis. Prenatal diagnosis was requested by ten pregnant women from hemophilia A families (Table 4). At least 1 of the 3 polymorphisms investigated was informative in all but 1 family. In this family, the fetus was male and the pregnancy was electively terminated. Carrier status was excluded by RFLP analysis in 2 women. In the remaining 7 pregnancies, 2 fetuses were determined to be female and no further analysis was carried out. In the remaining 5, prenatal diagnosis was accomplished by RFLP analysis in 3, using the St14 probe in 2 and the DX13 probe in 1. DNA analysis predicted a normal and 2 affected fetuses, and the diagnoses were confirmed by fetal or cord blood analysis. In the remaining 2 cases with male fetuses, chorionic villous biopsy did not yield a sufficient amount of DNA for analysis. Prenatal diagnosis of an affected fetus was accomplished by fetal blood analysis in 1 case; in the other case, fetoscopy was unsuccessful and the parents decided to continue the pregnancy.

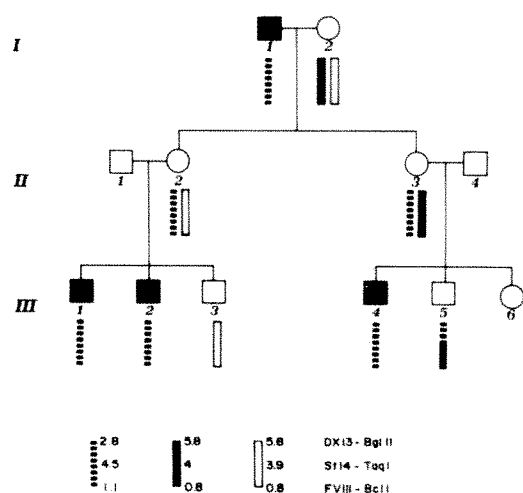


Fig 2. Pedigree, illustrating a crossing-over between the DX13 and the St14/FVIII loci. Bars: three chromosome haplotypes and the length of the restriction fragment length polymorphisms (RFLPs) of these three loci (bottom). In III-5, a recombination occurred between DX13 and St14/FVIII.

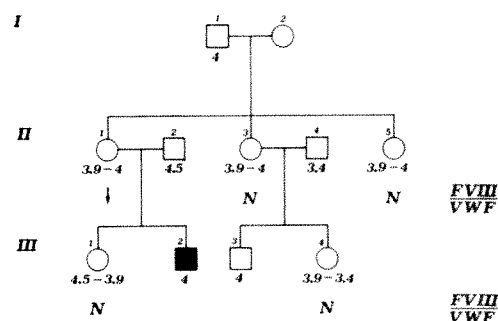


Fig 3. Pedigree illustrating a de novo occurrence of hemophilia A mutation. Numbers below the symbols denote the alleles of the St14 locus. Factor VIII biologic activity was measured by the FVIII/von Willebrand factor (vWf) ratio in the available females. These ratios were normal (N) in all except in II-1, in which it was decreased. Thus, the X chromosome with the 4-kb St14 allele that she passed to the affected son (III-2) already carried the hemophilic mutation. Because III-3 did not have hemophilia and II-3 is not a carrier, the mutation must have occurred in the grandfather (I-1).

Table 4. Prenatal Diagnosis of Hemophilia by DNA Analysis

Pregnancies monitored	10
Informative	9
RFLP analysis	
Noninformative	1 (Male, aborted)
Exclusion of carrier status by	
RFLP analysis	2
Sex determination of the fetus	
F	2
M	8
Diagnosis by RFLP analysis	3 (2 by <i>TaqI</i> -St14 1 by <i>BglII</i> -DX13)
Failures	2

DISCUSSION

This study shows that a combination of three DNA probes—factor VIII, St14, and DX13—will permit detection of the carrier status in the most hemophilia A families in which at least two subjects are affected in separate sibships (group I in Table 3). Use of the factor VIII, St14, and DX13 probes alone or in various combination allowed detection of 96% of the carriers. In families with sporadic patients of hemophilia A, when informative family members were available, RFLP analysis allowed exclusion of the carrier state in 86% of female relatives (Table 3). Despite this, RFLP analysis can be used for carrier identification only when the mother of the sporadic patients has been identified as a carrier on the basis of the conventional factor VIII assay.

RFLP analysis enabled us to establish a de novo occurrence of the hemophilia A mutation in the maternal grandfather in 7 of 12 (58%) families of sporadic cases in which members of three generations were available for analysis. This indicates a high frequency of spontaneous mutation of the hemophilia A gene, in agreement with previous estimates.^{32,33} This study clearly indicates that carrier detection and prenatal diagnosis require testing of the mother, brother, affected and nonaffected siblings, as well as maternal grandparents and, whenever possible, the father. *BclI* polymorphisms within the factor VIII gene should be used primarily for carrier detection and prenatal diagnosis of hemophilia A because misdiagnosis caused by recombination between this polymorphic site and the hemophilia gene is negligible. In our experience, the intragenic *BglII* polymorphism was not useful since it did not increase the information obtained using the *BclI* polymorphism. Investigation of other polymorphisms in the factor VIII gene, particularly the recently discovered *XbaI* polymorphism within intron 22,¹⁴ should be

a useful addition to the available markers. In families in which the polymorphic sites within the factor VIII gene are noninformative, linkage analysis with St14 and DX13 probes, closely linked to the hemophilia A locus, can be applied. In this study, the St14 locus showed no recombination with the factor VIII gene, whereas one recombinant was observed between the DX13 locus and the factor VIII/St14 loci. Previous studies have shown two crossovers between DX13 and the factor VIII gene¹⁸⁻²² and one between the DX13/St14 loci and the factor VIII gene.¹⁷ These results allow us to order the three loci along the X chromosome as follows: DX13/St14/factor VIII. In this study, the 95% confidence interval for probability of a recombination between the factor VIII locus and the St14 probe was 6.4% and that between the factor VIII locus and the DX13 site was 17.4%. Use of these random probes, particularly the DX13 probe thus carries a small risk of error, either in carrier detection or prenatal diagnosis. Despite this, DNA analysis for carrier detection has definite advantages as compared with the biologic assay, which is accurate in carrier identification in 70% to 95% of cases.³⁻⁶ Because of the risk of misdiagnosis, however, carrier detection by linkage analysis with extragenic polymorphic sites detected by random probes must be combined with the conventional factor VIII assay. Computer analysis may be used to calculate the genetic risk on the basis of pedigree information, RFLP segregation, and biological assay. Prenatal diagnosis of hemophilia A was accomplished successfully in 7 of the 10 cases investigated. Failures were caused by insufficient amount of chorionic villous DNA for analysis in two cases and uninformative RFLPs in one. From the data produced in this study, as well as in those already reported, we confidently predict that prenatal diagnosis of hemophilia A by linkage analysis with intragenic or extragenic polymorphic sites will be accomplished in ~95% of families in which a hemophilia A proband is available. According to recently published studies,³⁻⁶ ~30% of women at risk for being carriers of hemophilia A on genetic criteria have no living hemophilic male or father to track the mutant gene.³⁴ In these instances, conventional factor VIII assay of fetal blood will still be required in the future, even as more polymorphic sites within the factor VIII gene and or new random probes are identified.³⁵

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Immunoglobulin and T Cell Receptor Gene Configuration in Acute Lymphoblastic Leukemia of Infancy

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We examined immunoglobulin (Ig) heavy chain, κ light chain, and T cell receptor (TCR) γ and β gene configuration in the leukemic cells from a series of infants aged <1 year with acute lymphoblastic leukemia (ALL). Each of these 11 cases demonstrated leukemic cell surface antigens that have been correlated with a B cell precursor phenotype. Of the 11, lymphoblasts of 4 retained the germline configuration of both Ig and TCR loci, whereas 7 had rearranged the Ig heavy chain gene. Two of these seven showed light chain gene rearrangement. TCB β chain rearrangement had occurred in only one of the 11 patients' tumors. No TCR γ chain rearrangements were identified. These results are in contrast to earlier studies of B cell precursor ALL in

children in which Ig heavy chain gene rearrangements were evident in every case and ~40% showed Ig light chain rearrangement as well. In addition, 45% of cases of B cell precursor ALL of children had rearranged their γ TCR genes, and 20% had rearranged β . These data suggest that ALL in infancy represents an earlier stage of B cell development than is found in B cell precursor ALL of children. ALL in the infant age group has been associated with the worst prognosis of all patients with ALL. This study suggests that the disease in infants differs not only clinically, but also at the molecular genetic level, from the disease in children.

This is a US government work. There are no restrictions on its use.

RECENT TRIALS have found acute lymphoblastic leukemia (ALL) in infants aged <12 months to be distinct from ALL children with respect to its biologic and clinical features. Specifically, it is a more aggressive disease, with an increased incidence of leukocytosis, massive hepatosplenomegaly, central nervous system (CNS) involvement, thrombocytopenia, hypogammaglobulinemia, and less frequent remission inductions by day 14 of treatment.¹⁻⁸ We wished to determine the cellular lineage and state of maturation of the tumor cells of ALL of infancy, and to compare this with ALL of older children.

Immunoglobulin (Ig) genes and the antigen specific T cell receptor (TCR) genes provide molecular markers of lineage commitment, clonality, stage of differentiation, and malignancy-associated translocations for the ALLs of both B cell precursor and T cell types.⁹⁻¹⁴ Whereas ALL in children has been studied extensively using these tools,⁹⁻¹⁴ the configuration of Ig and TCR genes has not been characterized in ALL in infants. Recently, immunophenotypic analysis provided evidence for a B cell precursor origin of the disease in infants.¹⁵ In this study, we characterized ALL in 11 infants with respect to Ig and TCR gene status.

MATERIALS AND METHODS

Following informed consent of the parents, leukemic cells were obtained by Ficoll-Hypaque density-gradient centrifugation of pre-

treatment bone marrow aspirates containing at least 85% malignant blasts from 11 infants with the diagnosis of ALL.¹⁵ All infants were aged <12 months. Acute leukemias were initially defined as lymphoblastic based on French-American-British (FAB) morphology¹⁶ as well as phenotypic screening for B cell- and T cell-associated surface antigens by fluorescent microscopy as previously described.¹⁵ Monoclonal antibodies used included HLA-DR (4.1),¹⁷ B4 (CD19),¹⁸ Common ALL antigen (CALLA) (BA-3, CD10),¹⁹ and OKT11(CD2).²⁰ All leukemic cells were also examined for myeloid-associated antigens with the monoclonal antibodies L4F3,^{21,22} and a cocktail of 1G10(CD15),^{21,22} and 5F1(CDw14).^{21,22} Megakaryocyte differentiation antigens were examined with the 13.1¹⁵ and C7E10²³ monoclonals.¹⁵ All had been examined with the 12-8 monoclonal, which recognizes a 115-kd molecule present on hematopoietic colony-forming cells and their precursors.²⁴ At the time of this study, surface immunoglobulin (SIg) and 3A1²⁵ were also examined (Table 1). Patients were further characterized by sex, age in months, WBC and platelet counts at diagnosis, FAB morphology of lymphoblasts, and the presence or absence of meningeal disease, massive organomegaly, and relapse.

Immunoglobulin and TCR gene configuration. High-mol-wt DNA was extracted from leukemic cells as well as peripheral blood total WBCs from normal individuals. Ten micrograms of this genomic DNA was digested to completion with 3 to 5 U/ μ g of DNA of the restriction endonuclease *Bam*HI (Bethesda Research Laboratories) in all cases, and *Eco*RI (Bethesda Research Laboratories) and *Hind*III (Boehringer Mannheim) when ample DNA was available, size-fractionated on 0.8% agarose gels, and transferred to nitrocellulose paper.²⁶ Genomic blots were hybridized to ³²P-labeled DNA probes prepared by a nick-translation method,²⁷ washed at 52°C in 0.1 $\times$ 0.15 mol/L of NaCl/0.015 mol/L of NaCitrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), and autoradiographed. Blots hybridized with an entire TCR γ gene cDNA were washed at 42°C.^{28,29}

Human Ig gene probes were used to assess the status of immunoglobulin heavy (Ig H) as well as Ig κ light chain genes. A human genomic 6.0-kb *Bam*HI-*Hind*III fragment containing the entire Ig heavy chain joining (J_H) region was used to assess rearrangements of the Ig heavy chain locus.³⁰ This fragment detected a 17-kb *Bam*HI fragment in the germline form. This same fragment was examined in some samples using a 3' J_H probe, a genomic fragment of the J_H and 3' flanking region of the same human DNA. Ig H chain gene configuration was confirmed by hybridization of the 3' J_H probe with *Eco*RI- or *Hind*III-digested genomic DNA. κ gene rearrangements were also detected in *Bam*HI-digested genomic DNA by hybridization with a 2.5-kb germline *Eco*RI constant (C_κ) region-containing

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Table 1. Genotypes and Phenotypes of ALLs of Infants

Patient No.	IgH Chain	Genotype			Phenotype										
		κ L Chain	TCR γ	TCR β	HLA-DR	B4	CALLA	Slg	3A1	T11	L4F3	1G10/5F1	13.1	C7E10	12-8
1	R1	G	G	G	+	+	—	—	—	—	—	—	—	—	17
2	R1 D1	G	G	G	+	+	—	—	—	—	—	—	—	—	—
3	R2	G	G	G	+	+	15	—	—	—	—	—	—	—	—
4	G	G	G	G	—	32	36	—	—	—	—	—	—	20	—
5	R2	R1	G	G	+	+	—	—	—	—	12	—	—	—	—
6	G	G	G	G	20	11	+	—	—	—	—	—	—	—	—
7	R1	R1	G	R1	+	+	11	—	—	—	—	—	—	—	—
8	G	G	G	G	+	+	—	—	—	—	—	—	—	—	—
9	R1	G	G	G	+	+	—	—	—	—	—	—	—	—	—
10	R2	G	G	G	+	+	—	—	—	—	—	—	—	—	—
11	G	G	G	G	+	+	—	—	—	—	—	—	—	—	+

Phenotypic data (except for Slg and 3A1) were previously tabulated in Dinndorf and Reaman.¹⁵ The germline configuration of the Ig H chain locus was confirmed for patients 4 and 11 using *Hind*III and/or *Eco*RI digests in addition to *Bam*HI digests probed with the 3'J_H probe. The germline configuration of the γ TCR locus was found in every case by probing *Bam*HI-digested DNA with the C γ γ , J γ _{1,3}, and an entire TCR γ cDNA and was further confirmed by hybridization of *Eco*RI blots with the J γ _{1,3} probe for patients 4, 5, 10, and 11. The β TCR locus was studied by hybridization of *Bam*HI-digested DNA with the C β probe in all cases, and hybridization of *Eco*RI-digested DNA with the same probe for patients 4, 5, 10, and 11.

Numbers to the right of Genotype columns represent the number of alleles. — represents <10% of cells showing reactivity, + represents >50%, and specific values (percentages) are given for the 10% to 50% range.

Abbreviations: R, rearranged; D, deleted; G, germline.

fragment which recognizes a 12-kb *Bam*HI fragment in the germline context.³¹

TCR γ and β gene configurations were also assessed; γ gene configurations were examined by hybridization of *Bam*HI-digested DNA with each of three separate probes. The C γ γ probe, a TCR γ constant (C) region probe, was a 300-base pair (bp) *Bam*HI fragment of a human C γ 1-containing subclone³² (the kind gift of J. Seidman). When hybridized to *Bam*HI-digested human genomic DNA, the germline configuration of the C γ locus appears as a 15-kb fragment containing C γ 1, and a 12.5-kb fragment containing C γ 2. The configuration of the γ TCR locus in leukemic samples was further analyzed by hybridization of *Bam*HI-digested genomic DNA and *Eco*RI-digested genomic DNA with a TCR γ joining (J) region probe (J γ γ), a 700-bp *Eco*RI-*Hind*III fragment of a human genomic DNA containing the J γ _{1,3} subsegment³³ (the kind gift of T. Quertermous), and by hybridization of *Bam*HI-digested genomic DNA with a 1,500-bp *Eco*RI fragment containing an entire γ gene cDNA^{28,29} (the kind gift of T. Mak).

The C β probe, a 400-bp *Bgl*II cDNA fragment containing the human C β 2 region³⁴ (the kind gift of T. Mak), hybridizes to both C β regions under the stringency of hybridization described above. Configurations of the β TCR complex were assessed by hybridization of this probe with *Bam*HI-digested genomic DNA in all cases, and with *Eco*RI-digested DNA whenever possible. In the germline form, both J β -C β 1, and J β -C β 2 are located on a single 23-kb *Bam*HI fragment, which can be altered by rearrangements of either the C β 1 or C β 2 complex.

Statistical analysis. We examined whether significant genotypic differences existed between ALLs of infants aged <12 months and ALLs of children^{3,5} by Fisher's exact test.

In addition, clinical and biologic features of ALLs of infants and corresponding genotypic patterns were analyzed by either the Wilcoxon two-sample test or by Fisher's exact test when appropriate.

RESULTS

Ig gene configuration in ALL of infancy. Comprehensive genotyping of ALL of infancy reveals two patterns of Ig

gene configuration in the tumors in this series of patients; one with rearranged Ig genes (Fig 1A), and one with germline Ig genes (Fig 1B).

Two-thirds of the cases studied (7 of 11) have rearranged Ig H chain genes, and the majority of these (4 of 7) have rearranged both alleles (Fig 1A, Table 1). In one of these cases, rearrangement of one allele with deletion of the other is evident (patient 2). The leukemic cells of patients 1, 7, and 9 rearrange a single allele of the Ig H chain locus (Table 1). Two of the 7 with Ig H chain gene rearrangement have progressed to κ L chain gene rearrangement (Table 1).

The seven ALL cells that have rearranged Ig genes also express the B cell differentiation antigens HLA-DR and B4 and lack phenotypic evidence of T cell, myeloid, and megakaryocytic lineage (Table 1), suggesting that these cases represent precursors committed to the B cell lineage at both the genotypic and phenotypic levels. The majority of these leukemias (6 of 7), including the tumor cells of patient 5 with rearranged Ig H chain and κ light chain genes, are CALLA negative. The ALL cells of patient 7, which also have progressed to light chain rearrangement, have the B cell-associated surface antigens HLA-DR and B4 on the majority of cells as well as limited expression of CALLA (11%) (Table 1). Close examination reveals that the rearranged κ L chain band is less intense than the rearranged J_H band (Fig 2). Perhaps a subclone of the tumor cell population has developed this additional rearrangement. This population may overlap or may be identical to the population expressing CALLA.

TCR gene configuration in ALL of infancy. None of the 11 infants' leukemic cells in this series have rearranged γ TCR genes when analyzed by hybridization of *Bam*HI-digested DNA with the probes C γ γ , J γ _{1,3}, and an entire TCR γ cDNA. In 4 of the 11 cases (patients 4, 5, 10, and 11), the germline configuration of the γ TCR locus was further

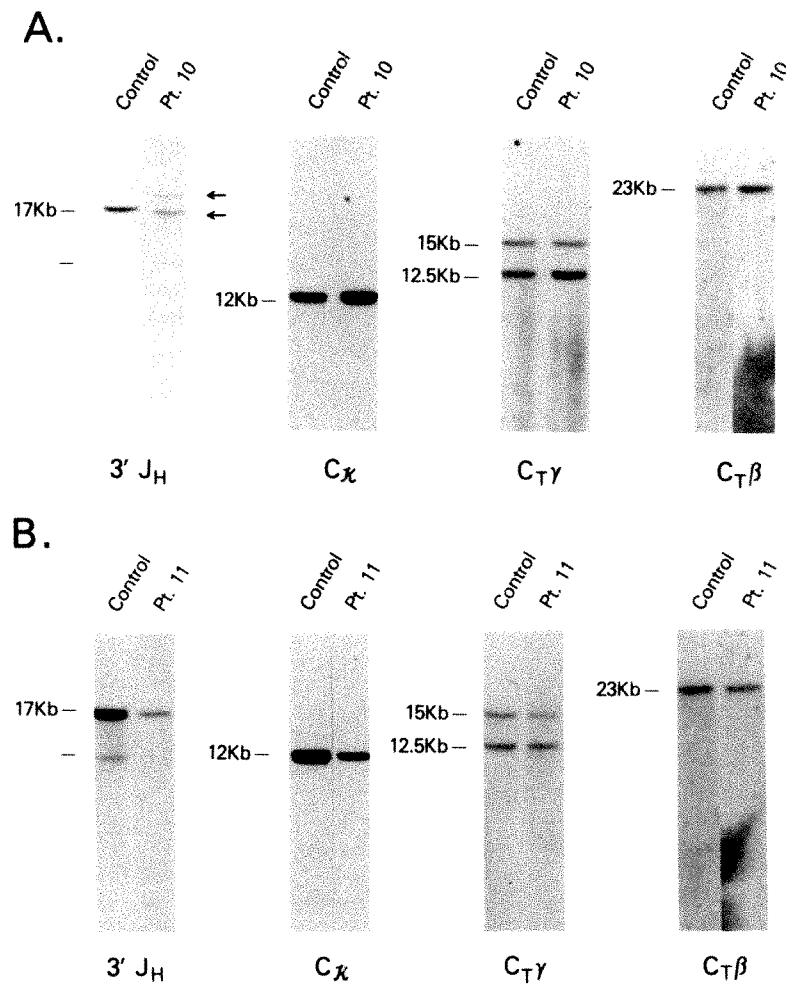


Fig 1. (A) The lymphoblasts of patient 10 show the pattern of rearranged Ig genes and germline TCR genes. In this case, both alleles of the Ig heavy chain are rearranged and the κ light chain gene remains in the germline form. Similar patterns of rearranged Ig genes were found in 7 of 11 ALLs of infants in the series examined. (B) The lymphoblasts of patient 11 show the germline configuration of the Ig heavy chain locus, and the κ light chain locus as well as the TCR γ and β genes. This pattern was found in 4 of 11 cases in the series of ALLs of infants examined.

confirmed by hybridization of *Eco*RI-digested DNA with the J_{γ1.3} probe (Figs 1A and B and 2; Table 1).

Similarly, ALL cells from 10 of the 11 infants have a germline configuration of the β TCR when *Bam*HI-digested DNA is hybridized with the C_Tβ probe (Fig 1A and B; Table 1). In 4 of these 10 cases, the germline configuration of the β TCR was confirmed by hybridization of *Eco*RI blots with the same C_Tβ probe (patients 4, 5, 10, and 11). The one exception (patient 7, Fig 2) has rearranged both Ig H chain and κ L chain genes, as well as the β TCR locus. These

leukemia cells, as mentioned above, demonstrate HLA-DR and B4 positivity and some CALLA, but lack any definitive T cell surface antigens, suggesting a B cell precursor lineage commitment.

ALLs germline for both Ig and TCR genes. One-third of ALLs of infancy (4 of 11) in this series have not rearranged either their Ig H chain, κ L chain, TCR γ , or TCR β genes (Table 1, Fig 1B). In two of these four cases (patients 4 and 11), ample DNA was available for confirmation of the germline configuration of the Ig H chain locus with the

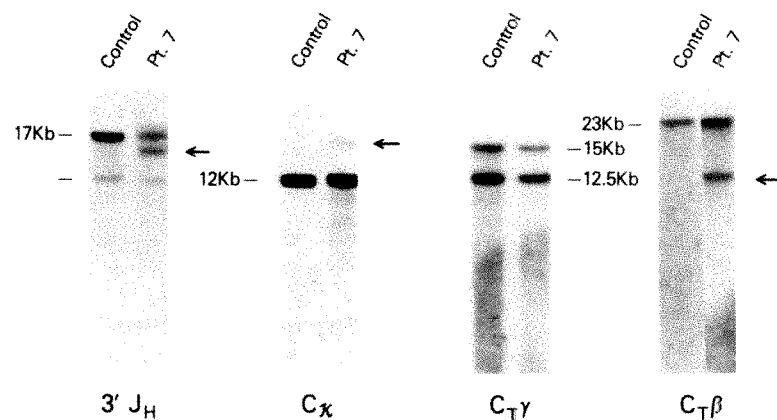


Fig 2. The lymphoblasts of patient 7 show rearrangement of the Ig heavy chain locus, and κ light chain locus and in addition show rearrangement of the β TCR locus. The rearranged κ light chain band is less intense than the rearranged J_H band, suggesting that a subclone of the tumor cell population has developed this additional rearrangement.

restriction enzymes *HindIII* and/or *EcoRI* in addition to *BamHI*. Each of these four leukemias expresses surface markers that have been correlated with early B lineage commitment (HLA-DR and/or B4). Two also express CALLA (patients 4 and 6). They lack the 3A1 and T11 T cell differentiation antigens, and do not express myeloid- or megakaryocyte-associated markers (Table 1). This pattern of surface markers and genes indicates that these cells may be very immature lymphoid precursors. Consistent with this, the tumor cells of patient 11 were positive when examined with the 12-8 monoclonal antibody, which recognizes a stem cell-associated antigen. This group with germline configuration of Ig and TCR genes suggests that the acquisition of the HLA-DR and B4 markers occurs early in lymphoid development, before the rearrangement of either Ig or TCR genes.

Comparison of genotypes of ALLs of infants and ALLs of children by statistical analysis. We examined whether genotypic differences existed between ALLs of infants aged <12 months and ALLs of children. By Fisher's exact test, the proportion with rearranged Ig genes was statistically significantly lower ($P_2 < .05$) in ALLs of infants (7 of 11) than in ALLs of children (25 of 25).¹⁰ In addition, the proportion of infants' ALLs with rearranged γ chain TCR genes (none of 11) was significantly lower ($P_2 < .05$) than in ALLs of children (8 of 17).¹²

We found no differences ($P_2 > .2$) between these two groups in the proportion with Ig heavy chain gene rearrangements that also rearranged light chains, nor in the proportion that rearranged their β TCR genes ($P_2 > .2$).^{10,12}

Correlation of genotype with clinical and biologic features of ALL of infancy. We tabulated clinical and biologic features and corresponding patterns of rearranged or germline Ig genes for this series of infants with ALL (data not shown). By the Wilcoxon two-sample test, there were no apparent differences in distribution of age in months, WBC, and platelet count at diagnosis or in percentage of blasts with L2 FAB morphology between infants with ALLs with germline ν rearranged Ig genes. Nor were there differences between infants with ALLs with germline ν rearranged Ig genes in sex, or proportions with meningeal involvement, massive organomegaly, or relapse when assessed by Fisher's exact test.

DISCUSSION

These data reveal that the clinical difference between ALL of infancy and ALL of childhood is correlated, in general, with distinctive patterns of Ig and TCR gene configurations. This may also be correlated with phenotypic differences in the leukemic infant population where CALLA positivity frequency is decreased.

Within this group of ALLs of infants, genotypic characterization suggests three subsets of lymphoid cells. One genotypic pattern is represented by a subset (4 of 11) that possesses germline configurations of both Ig and TCR genes. These cells express surface markers correlated with early B cell lineage (HLA-DR and/or B4), and lack surface antigens of other lineages, suggesting that they might be B cell precursors at a stage prior to Ig gene rearrangement. Inter-

estingly, one case of ALL in this subset (patient 11) reacts with the markers HLA-DR and B4, as well as the stem cell monoclonal 12-8, and may therefore represent a very early stage in lymphoid development. We have seen this germline pattern before in two children with ALL bearing some T cell-associated antigens,¹² another group in which a poor outcome often occurs.

Cells from only one infant in this series (patient 7) have rearranged their Ig genes as well as the β TCR locus. These cells may be examples in lymphoid development when both Ig and TCR genes are accessible to a putative common recombinase.^{35,38} This ALL is also interesting because it is an example of β chain rearrangement without γ . We have seen this pattern before in a rare T-ALL, as well as in some B cell precursor ALLs of children.¹² Whereas we found 45% of B cell precursor ALL cells of older children to possess rearranged γ TCR genes,¹² no γ TCR gene rearrangements were found in the 11 ALLs of infants in this group. This statistically significant finding indicates another molecular genetic difference between the disease in infants and the disease in children.

Finally, six of eleven ALLs of infants show rearranged Ig genes and germline TCR genes. Usually only Ig heavy chain genes rearrange without light chain gene rearrangement. These data suggest that ALL in infancy represents an earlier stage of B cell development than ALL of childhood.

The extensive phenotypic characterization of this group of ALL cells of infants has been presented before and is consistent with an early B lymphoid origin.¹⁵ Within the B cell precursor ALL series in children, a developmental hierarchy of Ig gene rearrangements in which all cases possess rearranged Ig heavy chains and ~40% had progressed to the later developmental step of κ followed by λ light chain rearrangement, was noted. This sequence was coordinate with an ordered expression of the B cell-associated surface antigens in which HLA-DR and B4 were present on the most immature cells, followed by CALLA and then B1 on the most mature cells.⁹⁻¹¹ In this context, we correlated genotypic and phenotypic data in our analysis of tumor cells from infants with ALL (Table 1). Based on this schema, we found several aberrant phenotypes. For example, CALLA expression that has been correlated with a stage of B cell precursor development after rearrangement of Ig heavy chain genes but before light chain activation was found on lymphoblasts of two infants with the germline Ig heavy and light chain gene pattern (patients 4 and 6), but not on the cells of another with rearranged κ L chain genes (patient 5). The tumor cells of patient 4 that express B4 and CALLA do not express HLA-DR. The cells of patient 6 were more positive for CALLA than for HLA-DR or B4. Thus, in this infant population, the order of Ig gene activation and coordinate B cell-associated surface antigen expression is not invariant. These "aberrant" phenotypes may reflect variability of normal lymphocyte development, assuming that differentiation in leukemic cells faithfully mimics normal differentiation. Alternatively, they may reflect events that result from malignant transformations.

The T cell phenotype has not been described in ALL in infancy and was not found in this series either. Its incidence

may be less than the 20% found in older children,^{39,40} as study of a larger series of infants with ALL suggested.¹

This molecular analysis of ALL in infancy extends our understanding of the genetic stages of lymphoid maturation in that it reveals cells with germline Ig and TCR loci that possess B cell-associated antigens and lack phenotypic markers of other lineages. Moreover, this study reveals ALL of infancy to generally represent an earlier stage of lymphoid

development than do the ALL cells of children. The distinctive molecular genetic patterns of this leukemia may be reflected in its biologic and clinical behavior.

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Erythropoietin Production by the Fetal Liver in an Adult Environment

By Alan W. Flake, Michael R. Harrison, N. Scott Adzick, and Esmail D. Zanjani

To gain insight into the mammalian liver to kidney erythropoietin (Ep) switch, we heterotopically transplanted livers from preswitch, switched, and postswitch fetal and newborn lambs into normal adult sheep. Recipients' serum Ep and circulating reticulocyte levels were serially determined until rejection of the graft and compared with identical samples from sham-operated control adult ewes. Transplantation of preswitch and switched fetal livers caused an impressive rise in recipients' serum Ep activity and provoked a corresponding increase in reticulocytosis. In contrast, Ep activity and reticulocyte counts did not change from preoperative levels in adult ewes transplanted with postswitch livers or in the sham-operated controls. The

production of Ep by the preswitch fetal liver in the adult environment was not dependent on the presence or absence of host kidneys and was stimulated by anemic hypoxia. These results suggest that (a) the fetal liver is capable of producing relatively large amounts of Ep activity, and (b) the production of Ep can be maintained in the adult environment in the presence of functional adult kidneys. This argues against suppression of liver Ep production by renal Ep, or some other factor in the postnatal environment, and suggests that the liver to kidney switch of Ep production during ontogeny may represent a genetically determined event.

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THE HORMONE erythropoietin (Ep) serves as the primary regulator of erythropoiesis in both fetal and adult life.^{1,2} The kidney is the major source of Ep activity in the adult.³ In contrast, the liver is believed to be the major source of Ep activity in utero.⁴ Most of the evidence for liver Ep production in utero is indirect, being derived from organ ablation studies in the sheep fetus. These studies suggest that the liver to kidney Ep switch is a gradual process beginning at ~130 days of gestation in the sheep (term 145 days) and concluding ~40 days after birth.⁵ The factors regulating this switch are not understood. Possible explanations include a genetically determined maturation process of the fetal liver or a suppressive factor in the adult environment that inhibits liver Ep production.

The fundamental stimulus in the fetus or the adult for Ep production is hypoxia.^{1,6} Similar degrees of hypoxemia provoke similar responses from either fetal liver or adult kidney.^{4,7} In the normal adult, extrarenal Ep production is minimal, and its role in the regulation of erythropoiesis is not known.⁸ The adult liver maintains its ability to produce Ep activity, however, and, in the renoprival adult, low levels of extrarenal Ep are essential for maintenance of marginal erythropoiesis.⁹ The adult liver, however, requires a much more severe hypoxic stimulus for Ep production than does the adult kidney.¹⁰ Therefore, between fetal and adult life, the threshold for hypoxic stimulation of liver Ep production increases.

We developed a model for the heterotopic transplantation of the fetal sheep liver into the adult neck (Fig 1). This model allowed us to address two questions: (a) Does the fetal sheep

liver produce Ep, ie, are conclusions drawn from indirect organ ablation studies correct? and (b) Can the fetal sheep liver maintain Ep production in the adult environment in the presence of functional adult kidneys?

MATERIALS AND METHODS

Experimental design. In all cases, surgery was performed under general anesthesia (Ketamine: 700 to 1,000 mg; halothane/oxygen). We transplanted livers harvested from 5 preswitch fetuses (105 to 120 days of gestation), 2 switched fetuses (> 135 days of gestation) and 3 postswitch lambs (45 days after birth) into the necks of adult sheep. In each case, the mother ewe was the recipient. Four other pregnant ewes served as sham-operated controls and underwent delivery by cesarean section, a complete neck dissection, and identical postoperative blood sampling. Recipient blood samples for reticulocyte counts and Ep activity were taken from a peripheral vein on the day of transplantation and every 2 days thereafter. Liver viability and rejection were monitored by percutaneous Tru-cut needle biopsy with each blood sample drawn.

Fetal liver harvest and preservation. Fetuses were delivered by cesarean section. The umbilical vein was cannulated, and the fetus was heparinized. The fetal heart was exposed by median sternotomy, and the suprahepatic inferior vena cava was severed at its junction with the right atrium, resulting in rapid fetal exsanguination. Simultaneously, perfusion of the umbilical vein with cold (0 to 4 °C) Ringer's lactate solution was begun and the umbilical cord was divided.

Perfusion of the umbilical vein with cold Ringer's solution was continued throughout the harvesting of the liver. The donor was opened longitudinally in the midline. The diaphragm was incised to the origin of the phrenic veins bilaterally, and these were ligated. The inferior vena cava was exposed above the renal veins. The portal vein and common bile duct were ligated, and the hepatic artery was dissected free in continuity with a long segment of donor aorta. The remaining ligamentous attachments of the liver were severed, and the liver was placed in an ice bath until transplantation. All livers were harvested and placed directly into the recipient's neck with cold ischemia times of <2 hours.

Fetal liver transplantation. A schematic representation of our model for heterotopic fetal liver transplantation is shown in Fig 1. During fetal liver harvest, the maternal laparotomy was closed and the neck dissection was begun. A linear incision was made over the recipient left external jugular vein, and skin flaps were raised. The external jugular vein and carotid artery were exposed. The fetal liver was oriented over the recipient jugular vein with the umbilical vein cephalad and the suprahepatic vena cava caudad in a linear arrangement. With ×3.5 magnification and standard vascular technique, we performed the following anastomoses: fetal aortic segment to recip-

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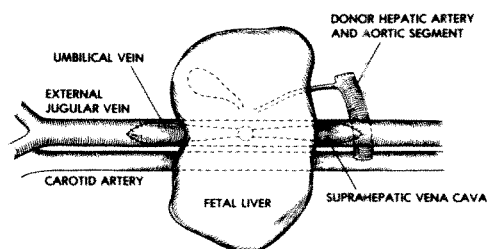


Fig 1. Schematic representation of the heterotopic fetal liver transplant model.

ient carotid artery; fetal suprahepatic vena cava to recipient jugular vein; and umbilical vein to the recipient jugular vein. This provided arterial inflow, outflow, and portal inflow, respectively. In post-switch lamb livers, umbilical vein involution required use of the portal vein for portal inflow. Cold umbilical perfusion was maintained until the last anastomosis was begun, minimizing warm ischemia. Bile was drained externally by a catheter in the gall bladder.

Nephrectomy and bleeding of recipients. To examine the effect of anemic hypoxia on Ep production by the transplanted liver in the host environment, two mother ewes were transplanted with pre-switch livers as described. On day 3 after transplant, the recipients were bilaterally nephrectomized through retroperitoneal incisions. The animals were allowed to recover for ~2 hours and then were rendered anemic by the removal of volumes of blood equivalent to 1.7% body weight. Plasma samples for Ep determinations were obtained before and after nephrectomy and at 6 hours after bleeding. Two nontransplanted adult ewes were similarly bilaterally nephrectomized and subjected to bleeding served as controls.

Ep assay. The Ep assays were performed in exhypoxic polycythemic mice (19 hours of hypoxia/day; total 219 hours). Serum samples (1 mL/mouse) were injected on day 5 posthypoxia, radio-iron (^{59}Fe -C13: 0.5 μCi /mouse) was administered on day 7, and the percentage of ^{59}Fe uptake was determined 72 hours later. The standard International Reference Preparation (IRP) Ep dose-response curves were established with each assay. Average control percentage RBC ^{59}Fe uptake values in these assays were: saline 0.62 ± 0.12 ; 0.05 IU Ep 2.20 ± 0.28 ; 0.1 IU Ep 5.15 ± 0.52 ; 0.4 IU Ep 11.38 ± 1.72 .

RESULTS

Function and rejection of the fetal graft. We have performed over 30 successful auxiliary fetal liver transplants with this technique.¹¹ The auxiliary grafts excrete 50 to 100 mL/day of bile for 5 to 7 days after transplantation, with

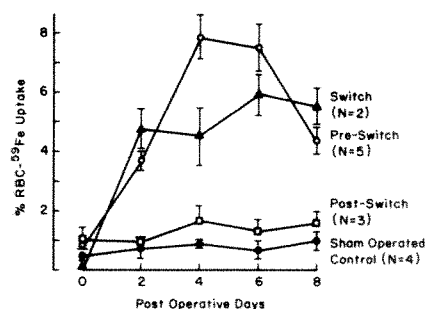


Fig 2. Production of erythropoietin (Ep) by the preswitch and switched fetal livers, but not by postswitch newborn livers, in the adult environment. Each value represents the mean $\pm$ 1 SEM of results from all animals in each experimental group. Plasma from each animal in the group was assayed in at least five polycythemic mice.

graft viability limited by rejection. At 24 to 48 hours after transplantation, adherence of lymphocytes to portal venous endothelium occurs (endotheliitis). By 72 hours, marked periportal infiltration by activated lymphocytes is present, and by 5 to 7 days the hepatic parenchyma becomes necrotic. This process can be delayed by immunosuppression, which was not used in this study. No difference in the sequence or histologic appearance of the rejection process was noted between the preswitch, switched, and postswitch grafts.

Ep production. Transplantation of the preswitch fetal livers caused an impressive rise in recipients' serum Ep activity and provoked a corresponding increase in reticulocytosis in the hosts (Fig 2 and Table 1, respectively). In all five animals, these values increased progressively until rejection of the graft. Similarly, transplantation of livers from switched fetuses resulted in increased circulating levels of Ep and reticulocytosis in the host animals. Although the relative increase in Ep activity in the latter group was somewhat less than that observed in the preswitch group (Fig 2), this difference was not statistically significant. In contrast, Ep activity and reticulocyte counts did not change from preoperative levels in the postswitch recipients or the sham-operated control animals.

Results shown in Table 2 demonstrate that at 3 days after the transplantation of preswitch livers the host circulation contained elevated levels of Ep. Bilateral nephrectomy alone did not alter the Ep levels in these recipients. Significantly

Table 1. Circulating Reticulocyte Levels in Sham Control and Adult Ewes Transplanted With Livers From Preswitch, Switched, and Postswitch Fetal Sheep and Newborn Lambs

Days Posttransplant	Reticulocytes (%)*			
	Control	Preswitch	Switched	Postswitch
0	0.015 ± 0.010 (n = 4)	0.024 ± 0.01 (n = 5)	0.01 ± 0.002 (n = 2)	0.006 ± 0.006 (n = 3)
2	0.013 ± 0.004 (n = 4)	0.200 ± 0.10 (n = 3)	0.15 ± 0.100 (n = 2)	0.010 ± 0.006 (n = 3)
4	0.010 ± 0.004 (n = 4)	0.310 ± 0.08 (n = 5)	0.32 ± 0.180 (n = 2)	0.015 ± 0.013 (n = 3)
6	0.020 ± 0.005 (n = 4)	0.210 ± 0.03 (n = 4)	0.22 ± 0.110 (n = 2)	0.011 ± 0.010 (n = 2)

*Each value represents mean $\pm$ 1 SEM of results from the number of animals (parentheses) in each group. Percentage of reticulocytes per determination were derived by enumerating 1,000 cells.

Table 2. Effect of Anemic Hypoxia on Erythropoietin Production in Bilaterally Nephrectomized Control and Liver Transplanted Ewes

Material Assayed	RBC- ⁵⁹ Fe Uptake (%)*	
	Animal 1	Animal 2
Sham-operated controls		
Plasma before nephrectomy	0.61 ± 0.08	0.90 ± 0.32
Plasma after nephrectomy	0.83 ± 0.22	0.88 ± 0.21
Plasma after bleeding	1.08 ± 0.32	1.42 ± 0.39
Transplanted animals		
Plasma before transplant	0.98 ± 0.38	0.55 ± 0.10
Plasma 3 days after transplant	6.84 ± 0.89	5.63 ± 0.76
Plasma after nephrectomy	7.06 ± 1.21	6.33 ± 0.84
Plasma after bleeding	19.69 ± 3.12	24.16 ± 2.72

*Determined in exhypoxic polycythemic mice (five mice/group).

greater production of Ep occurred, however, in these nephrectomized hosts at 6 hours following the induction of anemia by bleeding (Table 2). By contrast, bilaterally nephrectomized, nontransplanted adult ewes failed to exhibit significantly elevated Ep levels at 6 hours after bleeding (Table 2).

DISCUSSION

The absence of increased Ep activity in the sham-operated controls and recipients engrafted with postswitch livers demonstrates that the increases in Ep and reticulocyte production in animals transplanted with preswitch and switched livers were not the result of a host response to hypoxemic/anemic stress induced by the experimental procedure. This is in agreement with other studies in which we measured Ep and reticulocyte levels in adult sheep before and following a variety of surgical procedures; no detectable change in these parameters was observed. Moreover, the absence of increased Ep production in the recipients of the postswitch grafts precludes the possibility that the increases that occurred in the preswitch recipients are due to host Ep production in response to some factor released by the graft after transplantation or during rejection. Thus, the transplanted preswitch and switched fetal livers are clearly implicated as the source of the increased Ep activity. This was confirmed by the data shown in Table 2. Increased production of Ep occurred in bilaterally nephrectomized hosts engrafted with preswitch livers in response to bleeding. That the increase in Ep production in these recipients can be attributed to the donor livers and not to host sources was demonstrated by the fact that bilaterally nephrectomized adult control ewes failed to exhibit elevated Ep levels in response to a similar degree of anemic hypoxia.

Even after significant blood loss, reticulocyte increases in adult sheep are relatively small and, unless accompanied by

continued increased production of Ep, do not persist beyond 48 hours. The quantity of Ep produced by the fetal livers in this study is impressive. Conversion of the percentage of ⁵⁹Fe erythrocyte incorporation values into equivalent units of Ep, by comparison with dose-response curves established for the first International Reference Preparations of human urinary Ep, indicated peak values of >0.1 IU/mL of recipient serum. If one considers that the average adult sheep blood volume (3,000 mL) is 15 times greater than the blood volume of the average near-term fetal lamb (200 mL), this translates into a fetal Ep level of ~1.5 IU/mL (plasma samples obtained from donor fetuses at the time of liver harvest were devoid of detectable Ep activity by the polycythemic mouse assay). Direct measurement of fetal lamb Ep levels after hypoxic/anemic stress documented Ep levels as high as 2.5 IU/mL of fetal serum,¹² and in recent studies (E. D. Zanjani, unpublished observations), fetal lambs maximally stressed by bleeding and cobalt treatment produced Ep levels of >5 IU/mL of fetal serum, indicating that the fetal liver is capable of a significant Ep response to in utero hypoxic stress and that the fetal liver grafts were submaximally stimulated. These grafts retain their ability to produce additional Ep on further stimulation, however. This was demonstrated by the fact that the induction of anemia in the host by bleeding resulted in the production of significantly increased amounts of Ep by the donor liver. Why the transplanted livers were stimulated to produce Ep is unknown. Umbilical perfusion with adult venous blood (desaturated, low-affinity hemoglobin) may result in relative parenchymal hypoxia. All engrafted livers had normal histology with minimal alterations in the relative hematopoietic (primarily erythroid) cellular composition of the grafts, however, and produced large amounts of bile (50 to 100 mL/day) until rejection, suggesting that the parenchymal cells were unstressed. Whatever the cause, it is of interest that the stimulus was not adequate to induce Ep production by the postswitch lamb liver, suggesting that the threshold for stimulation of Ep production by the postswitch liver had already increased.

Explanations for the liver to kidney Ep switch around the time of birth include simple masking of the relatively small amount of liver Ep production by increased kidney Ep production after birth,¹³ or the suppression of liver Ep production by a substance made by the kidney, perhaps renal Ep itself.^{14,15} Our finding that the fetal liver produces large amounts of Ep activity in the adult environment, with functioning kidneys present, argues against a masking phenomenon, or the presence of a renal or other suppressive factor in the adult environment. The results we present therefore support the view that the liver to kidney switch of Ep production during ontogeny is a genetically predetermined event rather than a result of postnatal environmental factors.

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Effect of Monoclonal Antibodies Against von Willebrand Factor and Platelet Glycoproteins IIb/IIIa on the Platelet Retention Test

By Jean McPherson, Sandra Brownlea, and Marjorie B. Zucker

The platelet retention test provides a measure of the number of platelets retained in a column of glass beads and is one of the few *in vitro* platelet function tests that is abnormal in von Willebrand's disease (vWd). In a two-stage test, 1 mL of blood (designated A) was passed through the column, followed by 5 mL of isotonic saline and then 5 mL of blood (B) in which platelet retention was measured. With normal blood as A and B, retention is very high in all 5 mL of blood B. In the first stage, platelets adhere to the glass beads; this requires fibrinogen but not von Willebrand factor (vWf). The platelet-platelet adhesion in the second stage requires vWf, is dependent on release of ADP, and fails to occur if thrombasthenic platelets are tested. Retention was normal when blood from a patient with afibrinogenemia was used as blood B. We have now used monoclonal antibodies to elucidate further the mechanism of platelet retention. Five antibodies to different epitopes on vWf essentially abolished retention in the one-stage test and in the second stage of the two-stage test, but had no effect on the first stage. Thus, the entire vWf molecule must be free of antibody to function in the platelet-platelet adhesion of the second stage of this test. Binding of the

antigen-antibody complex to the platelet Fc receptor was not responsible, as Fab and F(ab')₂ fragments of one of the antibodies were as effective as intact antibody, and as neither heat-aggregated IgG nor a polyclonal antibody to plasma factor IX inhibited retention. F(ab')₂ fragments of 6D1, an antibody to platelet GP Ib that prevents binding of vWf to platelets, also inhibited the second phase of retention. An antibody that inhibits binding of fibrinogen and vWf to GP IIb/IIIa (LJ-CP8) inhibited both the first and second stages of retention, whereas LJ-P5, an antibody that inhibits only the binding of vWf to GP IIb/IIIa, caused slight inhibition of retention when normal or afibrinogenemic blood was used as blood B and was reported to cause only partial inhibition of ADP-induced platelet aggregation in this afibrinogenemic patient. The results suggest that vWf is altered during rapid passage of blood through the glass-bead column so that it attaches to GP Ib, exposing GP IIb/IIIa, which then binds the altered vWf or fibrinogen, either of which can induce platelet aggregation (platelet-platelet adhesion) and thus retention in the column.

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THE PLATELET retention test is an *in vitro* measure of platelet function that is abnormal in blood from patients with von Willebrand's disease (vWd).¹ The test provides a measure of the number of platelets retained when native or heparinized blood is pumped through a column of glass beads. Under appropriate conditions, retention is low in the first milliliter to emerge from the column, and increases progressively so that >80% of the platelets from the fourth and fifth milliliter are retained.² If flow is rapid, retention of platelets in vWd blood is low.^{2,3} Retention is also low when normal blood is disturbed.⁴ ADP is released during passage of the blood through the column and is required for normal retention; prior release of ADP is responsible for the inhibitory effects of disturbance.⁵⁻⁹ Monoclonal antibodies that react with platelet glycoprotein (GP) Ib¹⁰ or GP IIb/IIIa¹¹ inhibit retention, suggesting that binding of vWf to GP Ib and binding of fibrinogen to GP IIb/IIIa may be important.

We developed a two-stage test in which 1 mL of blood (blood A) was passed through the column, washed out with isotonic saline or plasma, and was followed by 5 mL of blood (B), in each milliliter of which retention was measured.¹²

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Under these conditions, retention was high in the first few milliliters of blood B, in contrast to the low values observed when blood A was omitted. The enhancement of retention in the first few milliliters of blood B depended on the presence in blood A of fibrinogen, divalent cations, and platelets able to bind fibrinogen, (ie, not thrombasthenic). Von Willebrand factor (vWf) was not necessary, and blood with platelets incapable of binding vWf (ie, from patients with Bernard-Soulier syndrome) functioned normally as blood A. Sustained retention in blood B required vWf, ADP, and divalent cations, and was abnormal in thrombasthenia. Retention was normal when blood from a patient with afibrinogenemia was used as blood B. Interpretation of this finding is complicated, however, by the observation that ADP caused normal aggregation of the platelets in this patient's heparinized platelet-rich plasma (PRP), although fibrinogen is usually believed to be essential for aggregation.¹³ Recent studies with monoclonal antibodies suggest that aggregation in afibrinogenemic PRP may result from binding of vWf rather than fibrinogen to GP IIb/IIIa.¹⁴

Monoclonal antibodies to vWf have been produced that selectively inhibit different properties of this molecule.¹⁵⁻¹⁹ Antibodies have also been produced that react with GP Ib, preventing vWf binding,¹⁰ and with the GP IIb/IIIa complex, preventing the binding of both vWf and fibrinogen or vWf alone after platelet stimulation.²⁰ We studied the effect of these antibodies on platelet retention in an attempt to increase our understanding of the mechanisms of platelet interactions in flowing blood.

MATERIALS AND METHODS

Platelet retention test. Columns were prepared as previously described,^{6,8} using 2 g of glass beads (diameter 0.45 to 0.5 mm) (Bacto Laboratories, Sydney, or 3M, Minneapolis), packed tightly into polyvinyl tubing, 3.5-mm ID. Columns were stored in a desiccator for at least 24 hours prior to use.

Healthy normal subjects who had not taken aspirin or related drugs for at least 1 week were used as donors, as well as a previously studied patient with afibrinogenemia^{13,21} (fibrinogen 3 to 10 $\mu\text{g}/\text{mL}$ plasma; $5 \mu\text{g}/10^9$ platelets). Blood was collected through a 19-gauge butterfly infusion set into 30- or 35-ml syringes containing sodium heparin (Weddel Pharmaceuticals, Sydney, or Upjohn, Kalamazoo, MI, final concentration 4 U/mL). Care was taken to avoid air bubbles, and the contents were mixed by "twirling." These precautions to avoid disturbance of blood were previously described.^{4,7} In some studies, an appropriate volume of either monoclonal antibody diluted in isotonic saline or sodium azide at a concentration equal to that in the antibody dilution was added. Antibody concentrations are given as microgram per milliliter of plasma, calculated by use of the hematocrit. Unless otherwise noted, antibodies were tested on at least three donors, with similar results. Figures 1 through 3 show typical experiments. Concentrations of azide up to 0.25 mmol/L in plasma had no effect on platelet retention.

Retention was measured between 10 and 40 minutes after blood collection. The syringe was twirled to mix the contents and placed in the holder of a perfusion pump (Harvard Apparatus, S Natick, MA). Blood was passed through the column at a speed of 6 or 8 mL/min, and each emerging milliliter was collected in 10 μL of 20% dipotassium EDTA. The microhematocrit was measured, PRP was separated in a Thrombofuge (Coulter Electronics, Hialeah, FL) or by sedimentation, and platelets were counted in a Coulter Model ZF or B. The percentage of retained platelets in each milliliter was calculated.

For the two-stage test, 1 mL of blood (A) was pumped into the column, followed rapidly by 5 mL of isotonic saline to flush the column. Then blood B was pumped through; the first milliliter, which was mostly saline, was discarded, and the platelets were counted in each of the succeeding 5 mL.

Monoclonal antibodies and other reagents. Dr J.J. Sixma provided antibodies to vWf prepared by Dr J.A. van Mourik as follows: RAg-35, RAg 38, and RAg 201 as IgG, and RAg Asc as ascitic fluid. Dr D. Meyer provided antibody vWf 9 from which we prepared Fab and F(ab')_2 fragments by methods previously described.^{22,23} References and descriptions of the effects of these antibodies are given in Table 1. Dr Z.M. Ruggeri sent us Fab' fragments of antibodies LJ-CP8 and LJ-P5 that react with the platelet glycoprotein complex IIb/IIIa.^{14,20} Dr B.S. Coller gave us antibody 6D1, from which we made F(ab')_2 fragments.¹⁰ Antibody solutions contained 15 mmol/L of (0.1%) NaN_3 . A polyclonal rabbit antibody to human factor IX was obtained from Dakopatt, Copenhagen. Heat-aggregated IgG was prepared according to the method of Reckel and associates.²⁵

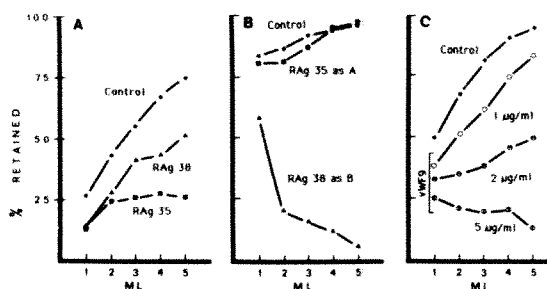


Fig 1. Effect of monoclonal antibodies against vWf on platelet retention. (A) One-stage tests: antibodies added at 1.2 $\mu\text{g}/\text{mL}$. RAg 201 and RAg Asc had the same effect as RAg 38. (B) Two-stage tests: RAg 35 added to blood A at 5 $\mu\text{g}/\text{mL}$; RAg 38 added to blood B at 3.3 $\mu\text{g}/\text{mL}$. RAg 201 and RAg Asc had the same effect as RAg 38. (C) One-stage tests: antibody vWf 9 added at different concentrations.

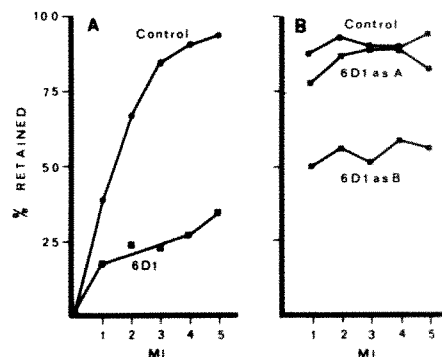


Fig 2. Effect of F(ab')_2 fragments of antibody 6D1 against GP Ib on platelet retention. (A) One-stage tests: 10.5 $\mu\text{g}/\text{mL}$ of 6D1. (B) Two-stage tests: 10.9 $\mu\text{g}/\text{mL}$ of 6D1 added to blood A or blood B.

RESULTS

In the one-stage test, all four antibodies from Sixma abolished platelet retention when added at concentrations of 3.2 to 3.6 $\mu\text{g}/\text{mL}$. At a concentration of 1.2 $\mu\text{g}/\text{mL}$, RAg-35 caused total inhibition of retention and the other three antibodies caused only partial inhibition (Fig 1A). In the two-stage test, none had any effect when added to blood A at 5 $\mu\text{g}/\text{mL}$, but all of them caused marked inhibition when added to blood B at 3.2 to 3.5 $\mu\text{g}/\text{mL}$ (Fig 1B). Antibody vWf 9 from Meyer also inhibited the one-stage test in a dose-dependent manner, with a maximal effect at 5 $\mu\text{g}/\text{mL}$ (Fig 1C). As with the Sixma antibodies, vWf 9 inhibited the two-stage test when added to blood B at 1 to 5 $\mu\text{g}/\text{mL}$, but was not effective when added to blood A at 5 $\mu\text{g}/\text{mL}$ (not shown). Fab and F(ab')_2 fragments of vWf 9 were as effective as whole IgG.

Polyclonal antibody to factor IX at concentrations up to 200 $\mu\text{g}/\text{mL}$ and heat-aggregated IgG at concentrations up to 500 $\mu\text{g}/\text{mL}$ had no effect on retention when used in a one-stage test or as blood A or B in the two-stage test.

F(ab')_2 fragments of 6D1 at 10.5 $\mu\text{g}/\text{mL}$ inhibited retention in the one-stage test in one donor (Fig 2A). In two other donors, they had no effect when added to blood A in the two-stage test (Fig 2B), but when added to blood B, inhibited

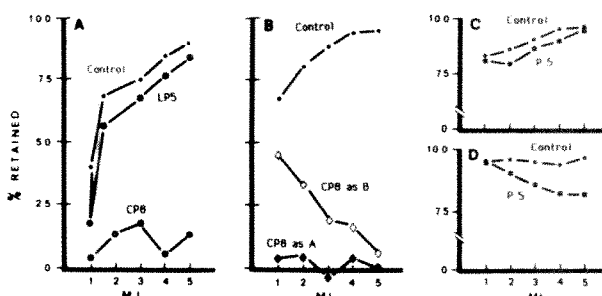


Fig 3. Effect of antibodies LJ-CP8 and LJ-P5 against GP IIb/IIIa on platelet retention. (A) One-stage tests: effects of 182 $\mu\text{g}/\text{mL}$ of LJ-P5 and 45 $\mu\text{g}/\text{mL}$ of LJ-CP8. (B) Two-stage tests, using 45 $\mu\text{g}/\text{mL}$ of LJ-CP8 in blood A or blood B. (C) Two-stage tests: normal blood alone or with 169 $\mu\text{g}/\text{mL}$ of LJ-P5 added to blood B. (D) Afibrinogenemic blood alone or with 133 $\mu\text{g}/\text{mL}$ of LJ-P5 added to blood B. Break in ordinate is apparent in C and D.

Table 1. In Vitro Effects of Monoclonal Antibodies Against vWF

Antibody			vWf-Dependent Function Affected												
			Platelet Agglutination With Ristocetin		Platelet Adhesion to Subendothelium		vWf Adhesion to Collagen		vWF Binding to Thrombin-Stimulated Platelets		Retention in Glass-Bead Column				
			Conc.* μg/mL	Inhib. (%)	Conc.* μg/mL	Inhib. (%)	Conc.* μg/mL	Inhib. (%)	Conc.* μg/mL	Inhib. (%)	Conc.† μg/mL	Inhib. (%)			
Designation	Source	Reference	RAg 35	Netherlands	15, 16, 17	1	100	10	100	24	0	NR		1.0	100
RAg 38	Netherlands	15	500	50	500	54	NR			NR				1.0	50
														3.2	100
RAg 201	Netherlands	15, 17	500	0	500	33	7	100	NR					1.0	50
														3.2	100
RAg Asc	Netherlands	—‡	500	0	500	0	500	0	NR					1.0	50
														3.2	100
vWF9§	France	18, 19, 24	100	10	20	95	NR			5	100			2.5	50
														5.0	100

NR, not reported.

*The inhibitory concentrations (Conc. Inhib.) of antibody are the lowest values given in the original articles but are not necessarily the minimum effective concentrations.

†Data from present study. Antibody concentrations represent lowest effective plasma concentration.

‡This antibody binds to vWf but does not affect its function (J.J. Sixma, personal communication).

§Fab and F(ab')₂ fragments were equally inhibitory.

retention partially (10.9 μg/mL, Fig 2B) or nearly completely (7.5 μg/mL, not shown).

Fab' fragments of antibody LJ-CP8 abolished retention when used at a concentration of 45 μg/mL in a one-stage test (Fig 3A) or as blood A or B in a two-stage test (Fig 3B). In the one-stage test, Fab' fragments of antibody LJ-P5 at 182 μg/mL caused very slight inhibition (Fig 3A); in other tests (not shown), however, retention was reduced by 16% to 20% with antibody concentrations of 86 and 169 μg/mL. Antibody had no effect when added to blood A and caused only minor inhibition when concentrations of 86 or 169 μg/mL were used in blood B (Fig 3C). When afibrinogenemic blood was used as blood B in the two-stage test, retention was as high as in normal blood, but antibody LJ-P5 (133 μg/mL) caused slightly more inhibition than it did in normal blood (Fig 3D).

DISCUSSION

The five monoclonal antibodies to vWf that we tested have different effects on the diverse functions of this large molecule (Table 1). They all inhibit platelet retention when added at low concentrations, however, including RAg Asc, which fails to affect any other activity of vWf (J.J. Sixma, personal communication). The antibodies affect only the second stage (blood B) of the two-stage test, which is the stage that requires vWf.¹² Ogata and colleagues²⁶ described four mouse monoclonal antibodies to vWf that inhibit platelet retention; only one of these inhibited ristocetin-induced agglutination.

Three observations provide evidence against the possibility that this lack of a differential effect resulted from nonspecific binding of antigen (ie, vWf)-antibody complexes to the platelet Fc receptor, known to be closely associated with GP Ib²⁷: (a) Fab and F(ab')₂ fragments of antibody vWf 9 inhibited retention as effectively as the whole antibody; (b) a polyclonal antibody to factor IX, a protein present in plasma at about the same concentration as vWf, failed to inhibit platelet retention; and (c) heat-aggregated IgG, which reacts

with the platelet Fc receptor in the absence of plasma,²⁸ had no effect on retention even at a very high concentration.

We and other researchers¹⁰ showed that antibody 6D1, directed against GP Ib, inhibits platelet retention. The dose-response relationship is not clear-cut, however. Coller and co-workers¹⁰ showed a reduction of retention from 95% to 48% in a one-stage test; greater inhibition could not be achieved by increasing the antibody concentration. We observed more marked inhibition (Fig 2A).

Although asialo vWf and vWf from patients with type IIB vWd bind to GP Ib without addition of ristocetin, normal vWf binds to GP Ib only in the presence of ristocetin.²⁹⁻³² Interaction of all three forms of vWf with platelets is prevented by monoclonal antibodies to GP Ib.^{10,28-31} The requirement for GP Ib in platelet retention suggests that passage of blood through the glass-bead column at high shear rate may alter either vWf or GP Ib in a way that results in their association. The observation that any of five monoclonal antibodies to different epitopes on vWf inhibits retention suggests that their attachment may prevent an alteration in vWf. Of interest is the recent report of a kindred with a variant form of vWd in which platelet retention was the only abnormal test. Plasma contained vWf that had retained the "pro" piece (ref. 33; T.S. Zimmerman, personal communication). These heterozygous subjects did not have a hemorrhagic diathesis.

Antibody LJ-CP8, which, like LJ-CP9,^{14,20} prevents binding of fibrinogen and vWf to the GP IIb/IIIa of activated platelets (Z.M. Ruggeri, personal communication), caused marked inhibition of both stages of retention. This is in accord with the abnormality of both stages of retention in thrombasthenic blood¹² and the inhibitory effect on the one-stage test of antibody 10E5 against GP IIb/IIIa.¹¹

Antibody LJ-P5 inhibits binding of vWf but not of fibrinogen to GP IIb/IIIa on activated platelets.²⁰ This antibody had only a slight inhibitory effect on the one-stage and two-stage tests in normal blood. According to De Marco and

colleagues,¹⁴ LJ-P5 has no effect on ADP-induced aggregation in normal citrated PRP but abolishes the moderate platelet aggregation that occurs in the citrated PRP of afibrinogenemic patients with plasma fibrinogen concentrations of $<8.5 \mu\text{g/mL}$. ADP-induced aggregation in these afibrinogenemic patients was increased by addition of vWf and was believed to be mediated at least in part by binding of vWf to GP IIb/IIIa; this binding does not occur in the presence of physiologic concentrations of fibrinogen.^{34,35} In our patient, ADP-induced aggregation was subnormal in citrated plasma, but normal in PRP anticoagulated with heparin.¹³ In agreement with our earlier findings,¹² retention was normal when his blood was used as blood B. Although LJ-P5 caused more inhibition than it did in normal blood when it was added to blood B, it was still slight, suggesting that the patient's fibrinogen concentration (3 to $10 \mu\text{g}/\mu\text{L}$) may be sufficient to support platelet-platelet adhesion in the second stage of platelet retention. This is supported by the finding that LJ-P5 did not completely inhibit primary ADP-induced platelet aggregation in citrated PRP from this patient (H.J. Weiss, personal communication).

vWf, but not fibrinogen, is essential for platelet aggregation in citrated blood or PRP that has been subjected to high

shear stress in a cone and plate viscometer.³⁶ vWf also plays a part in the formation of thrombi in native blood perfused at low shear rate over aortic endothelium^{37,38} and in the formation of occlusive thrombi in damaged coronary arteries in pigs.³⁹ Thus, events in a glass-bead column are relevant to important physiologic and pathologic processes. The mechanism by which vWf promotes platelet-platelet adhesion is not yet understood. Conditions in the glass-bead column resemble those in the circulation more closely than conditions in the usual *in vitro* studies of platelets, as the studies are carried out in whole blood with normal divalent cation concentration, and the disturbance caused by centrifugation⁷ is avoided. We hope that further studies will clarify the mechanisms.

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Inhibition of Heparanase-Mediated Degradation of Extracellular Matrix Heparan Sulfate by Non-anticoagulant Heparin Species

By Matia Bar-Ner, Amiram Eldor, Lina Wasserman, Yaacov Matzner, Irun R. Cohen, Zvi Fuks, and Israel Vlodavsky

Incubation of human platelets, human neutrophils, or highly metastatic mouse lymphoma cells with sulfate-labeled extracellular matrix (ECM) results in heparanase-mediated release of labeled heparan sulfate cleavage fragments ($0.5 < K_{av} < 0.85$ on Sepharose 6B). This degradation was inhibited by native heparin both when brought about by intact cells or their released heparanase activity. Degradation of heparan sulfate in ECM may facilitate invasion of normal and malignant cells through basement membranes. The present study tested the heparanase inhibitory effect of nonanticoagulant species of heparin that might be of potential use in preventing heparanase mediated extravasation of bloodborne cells. For this purpose, we prepared various species of low-sulfated or low-mol-wt heparins, all of which exhibited $< 7\%$ of the anticoagulant activity of native heparin. *N*-sulfate groups of heparin are necessary for its heparanase inhibitory activity but can be substituted

by an acetyl group provided that the *O*-sulfate groups are retained. *O*-sulfate groups could be removed provided that the *N* positions were resulfated. Total desulfation of heparin abolished its heparanase inhibitory activity. Heparan sulfate was a 25-fold less potent heparanase inhibitor than native heparin. Efficiency of low-mol-wt heparins to inhibit degradation of heparan sulfate in ECM decreased with their main molecular size, and a synthetic pentasaccharide, representing the binding site to antithrombin III, was devoid of inhibitory activity. Similar results were obtained with heparanase activities released from platelets, neutrophils, and lymphoma cells. We propose that heparanase inhibiting nonanticoagulant heparins may interfere with dissemination of bloodborne tumor cells and development of experimental autoimmune diseases.

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DISSOLUTION OF VASCULAR subendothelium occurs in vivo during processes such as inflammation and tumor invasion. Various observations suggest that the capacity of some bloodborne cells to extravasate may depend in part on their ability to express heparanase activity. Heparan sulfate (HS)-degrading endoglycosidases were described in various normal¹⁻⁵ and malignant⁶⁻⁹ cells. Most of these enzymes were endoglucuronidases,^{5,7} having an acidic pH optima but differing in their substrate specificities.⁷ Heparanase activity can be blocked in vitro by heparin,^{2-4,7,10} which is structurally related to HS but is less efficiently degraded by the enzyme.⁷ Heparin is a potent anticoagulant that also interferes with various physiologic processes such as smooth muscle cell proliferation,^{11,12} inflammation,¹³ angiogenesis,¹⁴ and autoimmune diseases.^{15,16} Some of the above activities of heparin were manifested by chemically modified heparin species which lack anticoagulant activity.¹¹⁻¹⁶ The broad spectrum of biological activities exerted by native heparin, most notably its anticoagulant properties, limits its application as heparanase inhibitor when in vivo studies are performed. Use can be made, however, of low-mol-wt heparin species or of chemically modified heparins devoid of anticoagulant activity to elucidate the involvement of heparanase in bloodborne cell egression.

The extracellular matrix (ECM) produced by cultured endothelial cells provides an appropriate model system for the subendothelium of blood vessels walls.^{17,18} Bovine endothelial cells grown in the presence of $\text{Na}_2[^{35}\text{S}]\text{O}_4$ produce ECM that contains HS as the major sulfate-labeled glycosaminoglycan (66% to 86% of the incorporated radioactivity).¹⁹ Human platelets³ and neutrophils⁴ as well as activated lymphocytes and macrophages^{1,2} degrade the ECM HS side chains into low-mol-wt fragments (K_{av} 0.63 to 0.7 on Sepharose 6B) by means of their heparanases. Expression of heparanase correlates with the metastatic potential of mouse melanoma,^{6,20} lymphoma,⁸ and fibrosarcoma⁹ sublines. The enzyme was also found in serum prepared from thrombin-treated platelet-rich plasma (PRP),³ in the supernate fraction of neutrophils incubated on ice,⁴ and in serum-free

medium conditioned by ESb lymphoma cells.⁸ Native heparin inhibited heparanase-mediated degradation of ECM by intact cells as well as by their respective cell-free enzyme preparations.¹⁰ The aims of the present study were (a) to evaluate the extent to which chemically modified or low-mol-wt heparins, that lack anticoagulation activity, retain the capacity of native heparin to inhibit heparanase-mediated release of HS fragments from subendothelial ECM; (b) to determine the minimal structural features of heparin required for inhibition of heparanase; and (c) to compare the inhibitory effect of native and modified heparins on heparanase elaborated by platelets,³ granulocytes,⁴ and metastatic lymphoma cells.⁸

MATERIALS AND METHODS

Fibroblast growth factor (FGF) was kindly provided by Dr D. Gospodarowicz, University of California, San Francisco. Dulbecco's

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modified Eagle's medium (DMEM, H-16), RPMI medium 1640, calf serum, fetal calf serum (FCS), penicillin, and streptomycin were obtained from GIBCO (Grand Island, NY). Trypsin-EDTA solution was obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes and flasks were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Some of the ECM-coated tissue culture dishes were kindly provided by International Biotechnologies (IBT, Jerusalem). Triton X-100 and dextran T-40 were from Sigma Chemical (St Louis). Thrombin was from Sigma, and sodium heparin was from Diosynth (Oss, Holland). Low-mol-wt heparins (main mol wt species 2,500 and 4,500 daltons), standard heparin, and synthetic α -methyl-glycoside pentasaccharide, representing the binding site of antithrombin III,²¹ were a kind gift of Dr J. Choay (Institut Choay, Paris). The two low-mol-wt heparin species, prepared by controlled depolymerization, are inactive against thrombin but have high factor Xa inhibiting activity.¹² The modified desulfated heparins were prepared as previously described.²²⁻²⁴ In brief, pyridinium heparin²² underwent exhaustive desulfation with dimethyl sulfoxide (Merck, Rahway, NJ) containing 10% water to yield totally desulfated heparin.²³ *N*-desulfated heparin was obtained from the respective pyridinium salt^{22,24} with dimethyl sulfoxide containing 5% water. Totally desulfated heparin and *N*-desulfated heparin were *N*-acetylated with acetic anhydride,²⁴ whereas resulfation of the free amino residues of the totally desulfated or *N*-desulfated heparin was performed with sulfur trioxide trimethylamine complex (Aldrich Chemical, Milwaukee) as described.²⁴ Cephalin in the form of activated cephaloplastin reagent was from American Dade (Aguada, Puerto Rico). $\text{Na}_2^{35}\text{S}\text{O}_4$ (540 to 590 mCi/mmol/L) was from New England Nuclear (Boston), and Lumax scintillation fluid was from Lumax Systems (Titusville, FL). Sepharose 6B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of reagent grade purchased from Sigma.

Endothelial cells. Cultures of bovine corneal endothelial cells were established from steer eyes as previously described.¹⁷⁻¹⁹ Cells were cultured in DMEM H-16 supplemented with 10% bovine calf serum, 5% FCS penicillin (50 U/mL) and streptomycin (50 $\mu\text{g}/\text{mL}$) at 37°C in 10% CO_2 humidified incubators. FGF (100 ng/mL) was added every other day during the phase of active cell growth.

Mouse lymphoma cells. A highly metastatic subline (ESb) of the methylcholanthrene-induced DBA/2 T lymphoma²⁵ was cultured in RPMI 1640 medium supplemented with glutamine (5 mmol/L, 2-mercaptoethanol (5×10^{-5} mol/L), streptomycin (50 $\mu\text{g}/\text{mL}$), penicillin (50 U/mL), and 10% FCS. A twice cloned line of ESb (clone 721) cells was established by limiting dilution and was used in the present study.⁸

Platelets. Blood from healthy individuals who had a history of no drug ingestion for at least 10 days before testing was obtained by venipuncture with two-syringe technique and mixed with 0.1 vol of 3.2% trisodium citrate.^{3,18} Platelet rich plasma (PRP) (2 to 3×10^5 platelets/ μL) was obtained from blood by centrifugation at 150 g for 10 minutes at room temperature. For preparation of washed platelets, PRP was centrifuged (1,100 g, 15 minutes, 20°C), and the pellet was washed twice and resuspended in acid-citrate-dextrose (ACD) buffer (pH 6.5), to yield the original concentration of 2 to 3×10^5 platelets/ μL .^{3,18}

Granulocytes. Granulocytes were prepared from fresh blood samples, obtained from healthy individuals, by dextran sedimentation followed by hypotonic lysis of contaminating erythrocytes and centrifugation over Ficoll-Hypaque, as described.⁴ The granulocyte pellet was washed in phosphate-buffered saline (PBS), free of Ca^{2+} and Mg^{2+} , and suspended at 5×10^6 cells/mL in PBS containing calcium chloride and magnesium chloride. Preparations obtained in this manner contained >95% granulocytes.⁴

ESb mouse lymphoma heparanase. Serum-free medium conditioned by ESb cells (3 to 5 days, 1×10^6 cells/mL) and prepared as described⁸ was used as a source for ESb heparanase activity.

Platelet heparanase. Thrombin (0.1 U/mL) was added to PRP prepared as described above. Following incubation for 2 minutes at 37°C, the thrombus was removed, and the remaining platelet-poor plasma (PPP) contained the released heparanase activity.³

Granulocyte heparanase. Granulocytes (5×10^6 cells/mL) isolated and suspended in PBS as described above were kept on ice for 60 minutes. The supernate containing heparanase activity⁴ was collected after removal of cells by centrifugation (300 g, 5 minutes, 4°C).

Preparation of sulfate-labeled ECM-coated dishes. Corneal endothelial cells were plated at an initial density of 4×10^4 cells/35-mm dish and maintained as described above except that 5% dextran T-40 was included in the growth medium.¹⁷⁻¹⁹ $\text{Na}_2^{35}\text{S}\text{O}_4$ was added twice (40 $\mu\text{Ci}/\text{mL}$), on the third day after seeding when the cells were nearly confluent, and 4 days afterwards, and the cultures were incubated with the label with no medium change. Ten to 12 days after seeding, the cell layer was dissolved by exposure (3 minutes, 22°C) to a solution containing 0.5% (vol/vol) Triton X-100 and 0.025N NH_4OH in PBS followed by four washes in PBS, leaving the underlying ECM intact, firmly attached to the entire area of the tissue culture dish and free of cellular debris.^{1-4,8}

Assay for heparanase activity. Heparanase activity assay using sulfate-labeled ECM as substrate was previously reported in studies with human platelets, human neutrophils, and ESb mouse lymphoma cells.^{1-4,8} In brief, cells or cell-free enzyme preparations were incubated (4 to 24 hours, 37°C, pH 6.2 to 6.5) with $^{35}\text{S}\text{O}_4$ -labeled ECM. Samples were then centrifuged (10,000 g, 5 minutes, 4°C), and 0.5-mL aliquots of the supernatants were applied for gel filtration on Sepharose 6B columns (0.7×35 cm) equilibrated with PBS containing 0.1% sodium azide. Fractions (0.2 mL) were collected at a flow rate of 5 mL/h and counted for radioactivity. The excluded volume (V_0) was marked by blue dextran, and the total included volume (V_T) was marked by phenol red. Similar gel filtration profiles (K_{av} values) were obtained by using the ECM produced by corneal or vascular endothelial cells, and whether the centrifuged media were subjected to gel filtration under dissociation conditions (4 mol/L of guanidine-HCl in 0.1 mol/L of sodium acetate pH 5.5) or eluted with PBS.⁸ Recoveries of labeled material applied to the columns ranged from 85% to 95% in different experiments. Each experiment was performed at least three times, and the variation in elution positions (K_{av} values) was <10%.

RESULTS

Modified and low-mol-wt heparins. Several modified heparins and low-mol-wt heparins of low anticoagulant activity were tested in the present study. The modified heparins were first either totally desulfated or *N*-desulfated. These heparins were then left with their N-position exposed or were further *N*-acetylated or *N*-resulfated. Although the unmodified heparin had an anticoagulant activity of 250 U/mg, and the totally desulfated heparin was devoid of any anticoagulant activity, the other modified heparins (*N*-desulfated; *N*-desulfated and *N*-acetylated; totally desulfated and *N*-resulfated; totally desulfated and *N*-acetylated) exhibited ~5% of the anticoagulant activity of native heparin (Fig 1 and Table 1). Two low-mol-wt heparins, one ranging from 1,500 to 8,000 daltons (main mol wt species 2,500 daltons) and the other ranging from 1,800 to 8,000 daltons (main mol wt species 4,500 daltons)¹² as well as a synthetic

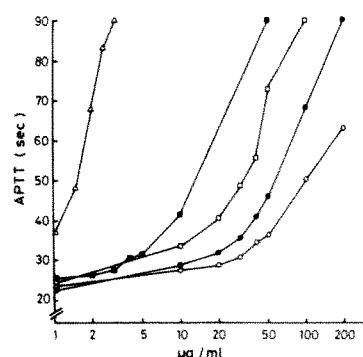


Fig 1. Dose dependence of coagulation inhibition activity of modified and low-mol-wt heparins. Activated partial thromboplastin time (APTT) was determined for various concentrations of standard heparin (Δ); totally desulfated and *N*-resulfated heparin ($\bullet$); *N*-desulfated and *N*-acetylated heparin ($\circ$); low-mol-wt heparin, 4,500-dalton main mol wt species ($\blacksquare$); or low-mol-wt heparin, 2,500-dalton main mol wt species ($\square$). APTT was performed with activated cephaloplastin reagent, using an automatic coagulation analyzer. APTT clotting time in the absence of any heparin was 20 seconds. Each data point represents the mean of four determinations and the standard deviation did not exceed 12% of the time measured.

α -methyl-glycoside pentasaccharide²¹ were also tested. The anticoagulant activities of these low-mol-wt heparin species were 17.5, 21.5, and 10.5 U/mg, respectively. For some of these heparin species, we tested dose dependence of coagulation-inhibiting activity. As shown in Fig 1, some of the heparin derivatives retained low anticoagulant activity even at high concentrations, whereas the two species of low-

mol-wt heparins effectively inhibited coagulation at high concentrations, despite their low anticoagulant activity at low concentrations.

Inhibition of platelet heparanase. Human platelets (2.7×10^4 platelets/ μ L), washed and suspended in ACD saline were incubated (37°C , 18 hours, pH 6.2) with sulfate-labeled ECM without or with either native heparin, *N*-desulfated and *N*-acetylated heparin, or heparin that underwent total desulfation followed by *N*-resulfation. HS degradation products released from ECM into the incubation medium were analyzed by gel filtration. Platelets cleaved the HS proteoglycan side chains into low-mol-wt fragments which eluted from Sepharose 6B at $K_{av} \sim 0.7$ (93% of the released radioactivity eluted in peak II, fractions 30 through 46). With native heparin (25 μ g/mL) only high-mol-wt labeled material was released (Fig 2) (radioactivity eluted in the region of peak II, fractions 25 through 40 was 6.5% of that observed in the absence of heparin, similar to that obtained in the absence of heparanase activity). With 10 mg/mL of native heparin, the amount of radioactivity eluted in peak II (fractions 30 through 46) was decreased by 85%, and the residual 15% degradation fragments had a higher mol wt than in the absence of heparin ($K_{av} \sim 0.5$ as compared to $K_{av} \sim 0.7$). Totally desulfated heparin added at the same concentration as native heparin did not exhibit any inhibitory effect on the platelet heparanase, as indicated by elution of >90% of the released radioactivity in the form of low-mol-wt material (peak II), similar to that released without heparin (Fig 2A). In contrast, totally desulfated heparin that underwent resulfation at the *N*-position inhibited by 51% heparanase mediated release of low-mol-wt labeled material (Fig 2A). *N*-desulfated and *N*-acetylated heparin inhibited even more effectively the cleavage of HS side chains, and the amount of radioactivity eluted in peak II was 30% of that eluted by platelets without any heparin, indicating 70% inhibition in the release of heparanase degradation products (Fig 2A). With both these modified heparins, the size of released HS fragments was larger ($K_{av} \sim 0.6$) than that observed in the absence of any heparin. Although native heparin, *N*-desulfated and *N*-acetylated and totally desulfated and *N*-resulfated heparins inhibited by 52%, 40%, and 31%, respectively, the total amount of radioactivity released from ECM by platelets, they inversely affected the amount of radioactivity eluted in the form of high-mol-wt material (Fig 2A).

The inhibitory effect of modified heparins was also demonstrated with the platelet heparanase in a cell-free preparation. In these experiments, platelet-depleted serum, obtained from thrombin-treated PRP, was incubated with sulfate-labeled ECM with or without the various heparins. Although native heparin (5.5 μ g/mL) completely inhibited the release of low-mol-wt HS fragments, both *N*-desulfated and *N*-acetylated heparin and totally desulfated and *N*-resulfated heparin, added at the same concentration, inhibited the release of low-mol-wt fragments by 50% to 60% (data not shown). Hence, the relative inhibitory effect of these modified heparins was similar when tested with either whole platelets or with their released heparanase.

Table 1. Inhibition of ESb Heparanase by Modified Heparins

Heparin Species	Anticoagulant Activity (U/mg)	Radioactivity in		Inhibition of Heparanase Activity (%)
		Peak I	Peak II	
No heparin	—	27	70	0
Unmodified heparin	250	83	13	100
Totally desulfated	0	24	73	0
<i>N</i> -Desulfated	16.5	31	66	7
Totally desulfated and <i>N</i> -resulfated	11.5	80	15	96
<i>N</i> -Desulfated and <i>N</i> -acetylated	11.5	82	14	99
Totally desulfated and <i>N</i> -acetylated	14.5	29	68	3
Heparan sulfate	ND	32	63	12

Serum-free medium conditioned by ESb cells was incubated with sulfate-labeled extracellular matrix (ECM) (37°C , 48 h, pH 6.2) without or with 5 μ g/mL of native heparin, heparan sulfate, or modified heparins, as indicated. The incubation media were collected, and labeled degradation products were analyzed by gel filtration over Sepharose 6B. Percentage of radioactivity in peak I ($K_{av} < 0.33$, fractions 15–27) and in peak II ($0.4 < K_{av} < 0.85$, fractions 31–47) was determined in relation to the total radioactivity released from the matrix by the ESb-conditioned medium in the presence of the heparin species tested. Heparanase inhibition was considered 100% with 5 μ g/mL unmodified heparin, and inhibition values for the other heparin species were determined in relation to native heparin.

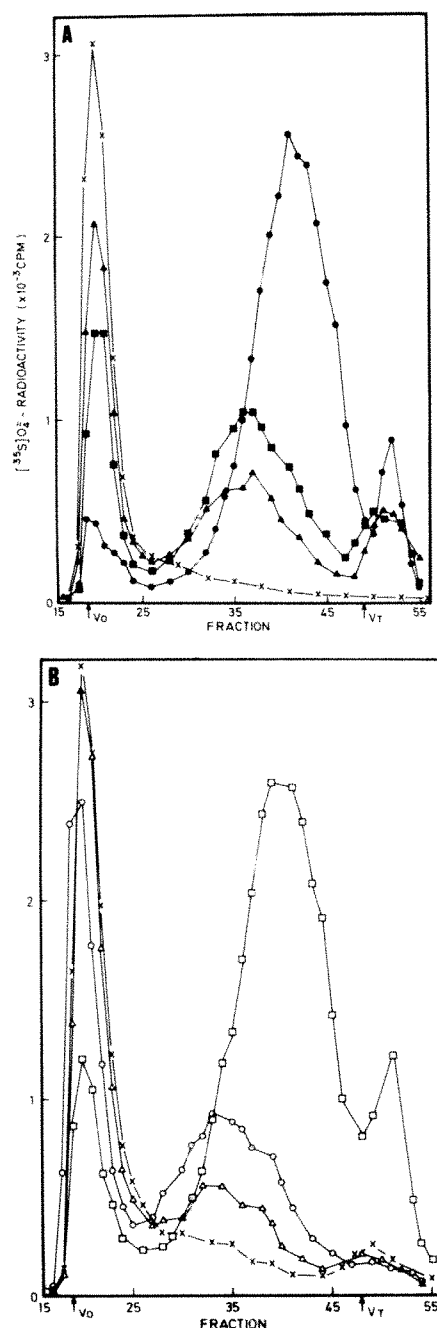


Fig 2. Effect of modified and low-mol-wt heparins on degradation of sulfate-labeled extracellular matrix (ECM) by human platelets. Human platelets (2.7×10^7 platelets/mL), washed and suspended in acid-citrate-dextrose (ACD) saline, were incubated (37°C , 18 hours, pH 6.2) with sulfate-labeled ECM without ($\bullet$) or with ($\times$) 25 $\mu\text{g/mL}$ of the relevant control standard heparin or (A) modified heparins: totally desulfated and *N*-resulfated heparin ($\blacksquare$); *N*-desulfated and *N*-acetylated heparin ($\blacktriangle$), or totally desulfated heparin (elution profile similar to that obtained with native heparin) ($\bullet$). (B) Low-mol-wt heparin, 4,500-dalton main mol-wt species (Δ); low-mol-wt heparin, 2,500-dalton main mol wt species ($\circ$); or synthetic α -methyl-glycoside pentasaccharide ($\square$). The incubation media were centrifuged, and the supernatants were analyzed by gel filtration on Sepharose 6B. The elution profile of labeled material released by the platelet heparanase with 25 $\mu\text{g/mL}$ pentasaccharide was similar to that obtained without heparin.

The inhibitory effect of low-mol-wt heparins on platelet heparanase is shown in Fig 2B. Platelets ($2.7 \times 10^4/\mu\text{L}$) washed and suspended in ACD saline, were incubated with sulfate-labeled ECM without or with each of the low-mol-wt heparins. At 25 $\mu\text{g/mL}$, both preparations of low-mol-wt heparins (main mol wt species 2,500 and 4,500 daltons) inhibited heparanase-mediated release of labeled fragments from ECM, albeit to a lower extent than did native heparin (Fig 2B). At higher concentration (50 $\mu\text{g/mL}$) inhibition of the platelet heparanase by low-mol-wt heparins was similar to that exerted by full-size unmodified heparin (not shown). The synthetic pentasaccharide exhibited only mild inhibition (10% to 20%) at 50 $\mu\text{g/mL}$ and none at all at 25 $\mu\text{g/mL}$ (Fig 2B).

Inhibition of neutrophil heparanase. Incubation buffer containing heparanase activity was derived from human neutrophils (2.5×10^6 cells/mL) kept on ice for 60 minutes. Incubation of this supernate with labeled subendothelial ECM resulted in release of low-mol-wt HS cleavage fragments which eluted with $K_{av} \sim 0.63$ from Sepharose 6B. In contrast, proteolytic degradation products released by the neutrophil supernate from ECM in the presence of native heparin (10 $\mu\text{g/mL}$) eluted from Sepharose 6B as a broad peak next to V_0 . Under this condition, 13% of the total released radioactivity eluted with $K_{av} > 0.5$ (Table 2), similar to that obtained without any heparanase activity (ie, pH 8). Inhibition of neutrophil heparanase by native and modified heparins had a pattern similar to that observed with platelets, yet lower concentrations were required for complete inhibition. Although totally desulfated heparin and the synthetic pentasaccharide failed to inhibit the neutrophil heparanase at 10 $\mu\text{g/mL}$, 86% inhibition of heparanase-mediated release of low-mol-wt labeled material was observed with the same concentration of *N*-desulfated and

Table 2. Inhibition of Neutrophil Heparanase by Modified and Low Molecular-Weight Heparins

Heparin Species	Radioactivity in		Inhibition of Heparanase Activity (%)
	Peak I (% of Total)	Peak II (% of Total)	
No heparin	23	59.4	0
Unmodified heparin	65.7	13	100
Totally desulfated and <i>N</i> -resulfated	51	25	75
<i>N</i> -desulfated and <i>N</i> -acetylated	57.7	19.8	86
Low-mol-wt, 2,500-dalton main species	63.9	15.5	95
Low-mol-wt, 4,500-dalton main species	66.4	13.4	99

Supernates of freshly isolated neutrophils were incubated (37°C , pH 6.2, 18 hours) with sulfate-labeled extracellular matrix in the absence or presence of 10 $\mu\text{g/mL}$ modified and low-mol-wt heparins, as indicated. The incubation media were collected, and labeled degradation products were analyzed by gel filtration over Sepharose 6B. Percentage of radioactivity eluted in peak I ($K_{av} < 0.33$, fractions 15–27) and in peak II ($0.5 < K_{av} < 0.85$, fractions 35–47) was determined in relation to total radioactivity released from the matrix. Heparanase inhibition was considered 100% with 10 $\mu\text{g/mL}$ of native heparin (described in text), and inhibition values for the other species of heparin were determined in relation to native heparin.

N-acetylated heparin, 95% to 99% inhibition with the two low-mol-wt heparins and 75% inhibition with totally desulfated and *N*-resulfated heparin (Table 2). When these heparins were tested at a lower concentration (2 $\mu\text{g}/\text{mL}$) for inhibition of a more concentrated heparanase preparation (supernatant derived from 8×10^6 neutrophils per mL), however, only native heparin completely inhibited heparanase-mediated degradation of the ECM HS.

Inhibition of ESb heparanase. Serum-free medium conditioned by highly metastatic ESb mouse lymphoma cells was incubated with sulfate-labeled ECM in the presence of chemically modified heparins. The inhibitory effect of totally desulfated and *N*-resulfated heparin and of *N*-desulfated and *N*-acetylated heparin was, at 5 $\mu\text{g}/\text{mL}$, essentially similar to that of native heparin (Table 1). Similar results were obtained with native heparin and with *N*-desulfated and *N*-acetylated heparin at 2 $\mu\text{g}/\text{mL}$, whereas at 1 $\mu\text{g}/\text{mL}$ release of low-mol-wt HS fragments was inhibited by 60% and 50%, respectively. In contrast, both *N*-desulfated heparin and totally desulfated and *N*-acetylated heparin failed to inhibit the enzyme, as demonstrated by the appearance of low-mol-wt (peak II) labeled products (Table 1). Hence, retention of *O*-sulfate groups is essential for the heparanase inhibitory activity of *N*-acetylated heparin. HS was a much less efficient inhibitor of heparanase than was native heparin or the other inhibitory modified heparins (Table 1). Complete inhibition of the ESb heparanase was not obtained even with a 20-fold higher concentration of HS (100 $\mu\text{g}/\text{mL}$) as compared with native heparin. Dermatan sulfate, at 50 $\mu\text{g}/\text{mL}$, completely inhibited release from ECM of low-mol-wt HS degradation fragments, whereas chondroitin sulfate failed to inhibit the ESb heparanase significantly at this concentration.

With the ESb enzyme, we also tested the effect of heparin fragments obtained through cleavage of commercial heparin and kindly provided by KabiVitrum AB (Stockholm). Preliminary results indicated that whereas disaccharides and trisaccharides were inactive as heparanase inhibitors at 50 $\mu\text{g}/\text{mL}$, oligosaccharides as small as tetrasaccharide inhibited release of low-mol-wt HS fragments from ECM, at this concentration. Decasaccharide, but not octasaccharide, however, exhibited a significant inhibition of the ESb heparanase already at 10 $\mu\text{g}/\text{mL}$ (data not shown).

DISCUSSION

In the present study, we showed that heparin species of low mol wt as well as some chemically modified heparins inhibited degradation of HS in the subendothelial ECM by heparanase activities derived from various cell types. This inhibition depended on the dose of heparin, and its effectiveness was related to the main size and certain chemical features of the heparin molecules. The inhibition also depended on enzyme concentration, but for a given heparin species the inhibitory capacity did not depend on the cell source of the heparanase, whether normal or malignant. In an attempt to mimic the complexity of the situation in vivo, we used a bioassay in which heparanase activity is measured by its capacity to degrade HS proteoglycans embedded in the

ECM deposited by cultured endothelial cells. Our results indicate that native and certain modified heparins inhibited the enzyme when expressed by whole cells incubated with this ECM, as well as crude preparations of enzyme released by cells and incubated with ECM. Native heparin was a more potent heparanase inhibitor than any of the modified heparins used. When these heparins were added at the minimal concentration sufficient for native heparin to inhibit the enzyme completely, only partial inhibition of heparanase was observed. When tested at higher concentrations, however, some chemically modified heparins completely inhibited degradation of the ECM HS. Similar results were obtained with the various cell types and heparanase preparations that were tested, whether cell associated or not.

Using the chemically modified heparin species, we were able to characterize some of the structural requirements for heparanase inhibition activity. Our results indicated that heparanase inhibition depended on the degree of sulfation of the heparin molecule, the position of sulfate groups, and the occupancy of the *N*-positions of the hexoseamines. Although totally desulfated heparin did not inhibit the enzyme, resulfation of the *N*-position reestablished its heparanase inhibitory activity, suggesting that a sulfate group at the *O*-position was not necessary for inhibitory effect provided that the *N*-position was sulfated. In contrast, *N*-acetylation of totally desulfated heparin did not result in heparanase inhibitory activity, indicating that under certain conditions the *O*-sulfate group was indeed required for enzyme inhibition. Partial desulfation of heparin that resulted in exposure of the *N* position, yet left the *O*-sulfates intact, caused complete loss of heparanase inhibitory activity. In this case however, if the desulfated *N*-position was substituted with the neutrally charged acetyl group, the modified heparin (*N*-desulfated and *N*-acetylated heparin) efficiently inhibited the three heparanases tested. Such heparin was also reported to inhibit melanoma heparanase.²⁶ In this modified heparin, the *O*-sulfate group had an important role since *N*-acetylation of totally desulfated heparin yielded a noninhibitory species for heparanase activity. A neutral charge at the *N*-position and intact *O*-sulfate groups were also necessary for the expression of antiproliferative activity on vascular smooth muscle cells by nonanticoagulant heparin.^{11,12} HS was about a 25-fold less efficient inhibitor of heparanase than native heparin or some of the nonanticoagulant heparin species. This could be due in part to the observation that heparin is a poor substrate of the enzyme as compared with HS.⁷

The heparanase inhibitory activity of heparin and its anticoagulant activity appeared to be unrelated. Low-mol-wt heparins showing <10% of the native heparin anticoagulant activity and certain chemically modified heparin species with <5% of the anticoagulant activity, retained substantial heparanase inhibitory activity. In contrast, a synthetic homogeneous pentasaccharide, representing the binding site in heparin for antithrombin III, had a very small or no heparanase inhibitory effect, dissociating these two activities as well. Heparin possesses several biological activities that are not related to its anticoagulant activity. Modified nonanticoagulant heparins retained the ability to inhibit vascular smooth muscle cell proliferation, angiogenesis, neutrophil chemotax-

is, and delayed-type hypersensitivity, and were suggested to be of potential therapeutic use.¹¹⁻¹⁵

Our studies with various types of normal bloodborne cells have demonstrated a correlation between expression of heparanase activity and the cell capacity to penetrate through blood vessel walls.¹⁻⁴ Expression of heparanase appeared to be an early event in T lymphocyte activation, one that could be induced by antigen^{4,29} and possibly other signals. Likewise, as found by other researchers and ourselves, the metastatic potential of mouse melanoma, fibrosarcoma, and lymphoma sublines correlated with their ability to degrade the ECM HS by means of their heparanases.⁷⁻¹⁰ Although the functional significance of heparanase activity in various normal and malignant cells is not yet clear, this enzyme, in conjunction with other hydrolytic enzymes,^{25,27,28} is likely to play a role in the solubilization of extracellular matrices and basement membranes. In this respect, heparinlike molecules, either present on the endothelial cell surface^{11,30} and in subendothelial ECM¹⁹ or, as previously suggested,¹¹ released in response to vascular injury, may modify the ability of cells to extravasate. Some indirect indications show that heparin and nonanticoagulant heparins may indeed affect lymphocyte traffic and inhibit the movement of cells through vessel walls. Administration of heparin prevented the disappearance of macrophages from peritoneal exudates, following intraperitoneal injection of antigen into sensitized guinea pigs.³¹ Sy and colleagues reported that the inhibition of hapten-specific delayed-type hypersensitivity by nonanticoagulant heparins was associated with reduction of the leuko-

cytic infiltrate and suggested that heparins may impair the emigration of inflammatory cells at the antigen challenge sites.¹⁵ We found that a daily injection of either native heparin or of heparanase inhibiting modified heparins markedly inhibited the progression of experimental autoimmune encephalomyelitis and adjuvant arthritis.¹⁶ Moreover, a similar treatment reduced the incidence of lung metastasis in tumor bearing experimental animals (manuscript in preparation). Likewise, Irimura and co-workers reported that preincubation of B16-BL6 melanoma cells with native and modified heparins greatly reduced the number of lung metastases following intravenous injection of the tumor cells to syngeneic mice.²⁶

Exogenously administered heparanase inhibitors such as nonanticoagulant heparins might be of potential therapeutic use in preventing the extravasation of normal and malignant cells in certain pathological situations. They can also serve as an important tool in elucidating the involvement of heparanase in cell invasion and migration during various processes such as inflammation, autoimmunity, tissue repair, and tumor metastasis, and in determining which of the biologic effects exerted by heparin is related to its heparanase inhibitory activity. Our in vitro experiments clearly indicate an inhibitory effect of nonanticoagulant heparins on degradation of HS in a naturally produced subendothelial ECM.

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ADP-Induced Platelet Aggregation Depends on the Conformation or Availability of the Terminal Gamma Chain Sequence of Fibrinogen.

Study of the Reactivity of Fibrinogen Paris I

By Marie-Hélène Denninger, Martine Jandrot-Perrus, Jacques Elion, Olivier Bertrand, Gene A. Homandberg, Michael W. Mosesson, and Marie-Claude Guillin

Fibrinogen Paris I contains a mutant γ chain that is longer than the normal chain, resulting in altered fibrin polymerization and cross-linking. Because these functions involve the carboxy-terminal region of the γ chain, we decided to determine whether fibrinogen Paris I or the isolated Paris I γ chain supports normal ADP-induced platelet aggregation, a function that requires the ultimate 12 residues of the normal γ chain (400 through 411). Aggregation of ADP-stimulated normal platelets was defective with fibrinogen Paris I and markedly depressed with the γ Paris I chain. These findings prompted us to characterize the carboxy-terminal structure of the region of the γ Paris I chain responsible for this activity. The carboxy-terminal cyanogen bromide (CNBr) peptide of the normal γ chain (385 through 411) or that from γ Paris I was isolated by differential adsorption to triethylene-tetramine resin or by reverse-phase high-performance liquid chromatography (HPLC). The CNBr peptide from the Paris I γ chain was

identical to that of the normal γ chain in its retention time on HPLC, its amino acid composition, and its sequence. Thus, the primary structure of the γ Paris I chain from residue 384 through 411 is normal, indicating that a peptide insertion has occurred upstream from residue 384, resulting in an impairment of those physiologic functions attributable to the carboxy-terminal end of the γ chain from position 384 (ie, cross-linking, ADP-induced platelet aggregation, and at least a portion of the γ chain polymerization site). These observations demonstrate that the γ chain platelet recognition site in the fibrinogen molecule is necessary but not alone sufficient to support normal ADP-induced platelet aggregation. There appears to be an additional requirement for normal conformation of the γ chain or availability of its terminal sequence during the interaction of fibrinogen with platelets.

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PLATELET STIMULATION by agonists such as ADP or epinephrine results in the exposure of specific and saturable binding sites for fibrinogen at the platelet surface¹⁻⁶ and leads to platelet aggregation. The fibrinogen receptors at the platelet surface have been identified as the noncovalent calcium-dependent complexes formed by glycoproteins IIb and IIIa.⁷⁻¹¹ Two sites of interaction have been identified on the fibrinogen molecule. One is located in the A α chain,^{7,12,13} and the other is located in the carboxy-terminal region of the γ chain. This latter site is contained within the sequence of the ultimate 12 amino acids (positions 400 through 411).¹⁴⁻¹⁶ The carboxy-terminal segment of the γ chain is also involved in other fibrinogen functions such as fibrin polymerization,¹⁷ cross-linking of fibrin by factor XIII,¹⁸ and staphylococcal clumping.¹⁹

Fibrinogen Paris I, isolated from the plasma of a heterozygote subject,²⁰ is characterized by the coexistence of normal

and elongated γ chains termed γ Paris I. Further studies demonstrated that γ Paris I chains^{21,22} do not participate in factor XIIIa-catalyzed cross-linking,^{22,23} do not incorporate dansyl-cadaverine in the presence of the enzyme,²² and produce an abnormal fragment D on plasmin degradation.^{23,24} These observations suggested that the structural defect might affect, either directly or indirectly, the carboxy-terminal region of the γ Paris I chains.

This prompted us to investigate the ability of γ Paris I chain to support ADP-induced platelet aggregation and to characterize structurally the carboxy-terminal region of the γ chain mutant.

MATERIALS AND METHODS

Materials

Guanidine hydrochloride ultrapure was purchased from Schwarz Mann, division of Beckton Dickinson (Orangeburg, NY), reagents used for polyacrylamide gel electrophoresis (PAGE), cyanogen bromide (CNBr), and iodoacetic acid from Eastman Kodak (Rochester, NY); Tris aminomethane (Tris), dithiothreitol (DTT), sodium dodecyl sulfate (NaDodSO₄), N-ethylmorpholine (NEM), Hepes, apyrase grade V, prostaglandin E₁ (PGE₁), bovine carboxypeptidase A (DFP treated) and standard L-amino acids were purchased from Sigma Chemical (St Louis); bovine serum albumin (BSA) fraction V from Miles (Elkhart, IN); aprotinin from Choay (Paris), ADP from Stago (Asnières, France); Sephadex G 10C from Pharmacia (Uppsala, Sweden); carboxymethylcellulose CM 23 from Whatman (Maidstone, England); Biorex RG 501-X8,DC-6A resin from Durrum (Palo Alto, CA), aquapore RP-300 from Brownlee (Santa Clara, CA); acetonitrile HPLC grade from Fisons (Loughborough, England); dimethylaminoazobenzene isothiocyanate (DABITC) and phenylisothiocyanate (PITC) from BDH (Poole, England); dansyl-chloride, aminopolystyrene resin, triethylenetetramine resin, dimethylformamide, triethylamine from Pierce Chemical (Rockford, IL); and polyamide sheets from Schleicher and Schuell (Dassel, FRG). When unspecified, chemicals were reagent grade or better.

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Proteins and Peptide Isolation

Blood from the propositus and normal donors was collected in 0.13 mol/L of sodium citrate containing 560 KIU/mL aprotinin (1 vol of anticoagulant/9 vol of blood).

Fibrinogen. Fibrinogen was isolated from plasma as previously described.²⁵ Fibrinogen concentrations were determined from absorbance reading at 280 nm assuming an extinction coefficient of 1.52 mL/mg/cm.²⁶

Normal fibrinogen γ chain. Normal fibrinogen γ chain was isolated as follows: after dialysis against 50 mmol/L Tris, 0.1 mol/L of NaCl, pH 7.4, fibrinogen was reduced and *S*-carboxymethylated.²⁷ The $\alpha\alpha$, $\beta\beta$, and γ chains were separated by chromatography on CM 23 cellulose²⁸ using deionized urea²⁹ for the gradient solution consisting of 8 mol/L of urea, 0.1 mol/L of acetic acid, buffered at pH 4.8 to 5.8 with Tris. The elution profile was as previously described.^{30,31} Purity of the normal γ chain was ascertained by NaDodSO₄ PAGE analysis.³²

γ Paris I chain. The inability of γ Paris I chain to cross-link in the presence of factor XIIIa was turned to an advantage for its purification, as described by Stathakis and colleagues.³³ In brief, cross-linked fibrin Paris I was prepared by recalcification of plasma; all the normal γ chains in the clot underwent covalent cross-linking to γ -dimers, whereas γ Paris I chains did not. The fibrin clot was then reduced, *S*-carboxymethylated,²⁷ and sieved on Sephadex G 100 (column 2.5 $\times$ 90 cm, 9.4 mL/h) equilibrated in a freshly deionized 8 mol/L of urea, 0.1 mol/L of CH₃COOH solution, buffered at pH 5.0 with Tris. Further purification of the γ Paris I chain was achieved by chromatography on CM 23 cellulose, as described above.

CNBr peptides from the normal and Paris I *S*-carboxymethyl γ chains. CNBr peptides were obtained by CNBr cleavage of the methionyl bonds, in 70% vol/vol formic acid, using CNBr in a 120-fold molar excess with respect to the methionyl residues. After 24 hours at room temperature, the digests were diluted 1:10 with water and lyophilized. The carboxy-terminal CNBr peptide was isolated by the method of Horn,³⁴ as recently applied to the preparation of the carboxyl-terminal CNBr fragment of rat fibrinogen gamma chains.³⁵ In brief, TFA (200 μ L) was added to the CNBr digests (200 μ g). After incubation for 1 hour at 22°C, the solutions were dried under a stream of nitrogen. Dimethylformamide (0.5 mL) was then added to the residues, followed by a slurry of 100 mg of aminopolystyrene and triethylenetetramine resin. After the addition of triethylamine (100 μ L), followed by 12 hours of stirring at 45°C, the suspensions were centrifuged and the supernatant containing the carboxy-terminal peptide was freeze-dried. The carboxy-terminal CNBr peptide was also purified by HPLC on an aquapore RP-300 column (0.46 $\times$ 25 cm). The gradient used was acetonitrile in 0.3% trifluoroacetic acid (TFA), and was increased linearly from 0% to 25% vol/vol acetonitrile in 15 minutes, then to 33%, 38%, and 42% vol/vol acetonitrile in 50, 15, and 60 minutes respectively, at a flow rate of 1.5 mL/min. The absorbance was monitored at 220 nm.

Amino Acid and Sequence Analysis

Amino acid analyses were performed by subjecting the peptides to hydrolysis in distilled 6 N HCl for 22 hours at 110°C under vacuum. Amino acids were identified by chromatography on a DC-6A column using an automatic amino acid analyzer (Liquimat 2, Labotron, Kontron Instruments, Zurich); amino acid detection was achieved with *O*-phthalaldehyde.³⁶

The carboxy-terminal residue was identified as follows: the γ Paris I chain (200 μ g) was dissolved in a 0.1 mol/L *N*-ethylmorpholine acetate buffer, pH 8.1, and 2 μ g of carboxypeptidase A (previously dialyzed against 10,000 vol of buffer) was added. After 1

hour at 37°C, the solution was freeze-dried, dissolved in 50 μ L of 0.5 mol/L of Na bicarbonate buffer, pH 9.8, containing 1% (wt/vol) NaDodSO₄, subjected to dansylation, and the carboxy-terminal amino acid was identified by both TLC³⁷ and HPLC.³⁸

Amino-terminal amino acid sequencing was performed by the manual liquid-phase DABITC/PITC procedure³⁹ or alternatively on an automatic gas-phase microsequencer from Applied Biosystems (Foster City, CA). The PTH derivatives were analyzed by HPLC.⁴⁰

Platelet Aggregation

Platelet-rich plasma (PRP) was prepared from human blood collected into acid-citrate-dextrose (ACD-A) (6 vol of blood/1 vol of anticoagulant), freshly drawn from healthy volunteers who had abstained from taking aspirin or other medication during the 10 preceding days. ACD-A (10% vol/vol), apyrase (25 μ g/mL), and PGE₁ (100 nmol/L) were added to PRP. The platelet pellet obtained by centrifugation for 15 minutes at 1,500 g at 20°C was resuspended in a washing buffer⁴¹ consisting of 36 mmol/L citric acid, 5 mmol/L of glucose, 5 mmol/L of KCl, 2 mmol/L of CaCl₂, 1 mmol/L of MgCl₂, 103 mmol/L of NaCl, pH 6.5, 25 μ g/mL of apyrase, 100 nmol/L of PGE₁. Platelets were separated by centrifugation for 20 minutes at 1,500 g through a stepwise discontinuous gradient⁴² consisting of 8%, 10%, 13%, 20%, and 40% albumin in the washing buffer. The platelet fraction was collected onto the 40% albumin cushion, washed twice in the washing buffer, and then resuspended in a reaction buffer consisting of 137 mmol/L of NaCl, 2 mmol/L of KCl, 12 mmol/L of NaHCO₃, 0.3 mmol/L of NaH₂PO₄, 2 mmol/L of CaCl₂, 1 mmol/L of MgCl₂, 5 mmol/L of glucose, 5 mmol/L of Hepes, pH 7.4, 0.35% BSA. The platelet count was adjusted to 3×10^8 /mL, and the platelets were used within 2 to 3 hours after blood collection.

Platelet aggregation was studied by measuring light transmission through a suspension of platelets stirred at 1,100 rpm in a Coulter aggregometer (Coulter Electronics, Hialeah, FL). Purified fibrinogen or γ chains (60 μ L) dissolved in 50 mmol/L of NH₄HCO₃, pH 7.8 (0.25 to 4 mg/mL) were added to 300 μ L of platelet suspension, and aggregation was induced by the addition of 40 μ L of ADP (final concentration 5 μ mol/L). Platelets prepared as described above did not respond to ADP without added fibrinogen.

RESULTS

Purification of γ Paris I Chains

When reduced and *S*-carboxymethylated cross-linked fibrin Paris I was subjected to gel filtration on Sephadex G 100, the chromatogram consisted of a single broad peak, with only a partial purification of the γ Paris I chain. Further purification on CM 23 cellulose yielded pure γ Paris I chain, which exhibited a 3,000-dalton difference in molecular mass when compared with normal γ chain by NaDodSO₄ PAGE (Fig 1).

ADP-Induced Platelet Aggregation in the Presence of Fibrinogen Paris I or γ Paris I Chain

In the presence of normal fibrinogen, aggregation of normal platelets is readily induced by the addition of ADP (Fig 2). When normal fibrinogen was replaced by equal amounts of fibrinogen Paris I, the rate and extent of platelet aggregation were reduced by approximately twofold, and the initial aggregation was followed by disaggregation.

As previously reported by Hawiger et al,¹² isolated normal γ chains support ADP-induced platelet aggregation (Fig 3,

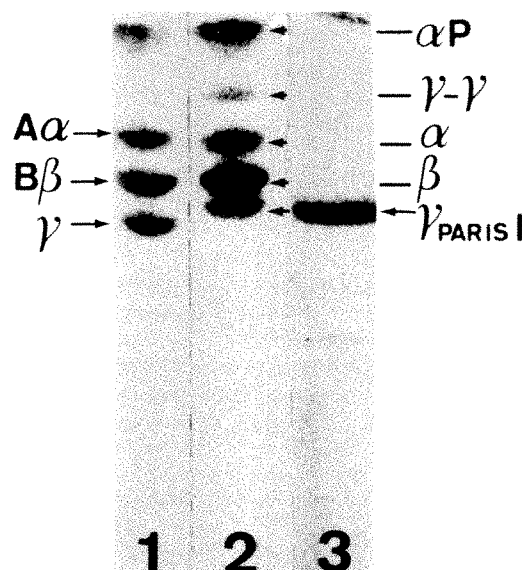


Fig 1. Gel electrophoretic analysis of γ Paris I chain. Samples of purified normal fibrinogen (lane 1), cross-linked fibrin Paris I (lane 2) and γ Paris I chain isolated on CM23-cellulose (lane 3) were reduced and subjected to electrophoresis in 10% acrylamide, 0.1% NaDodSO₄.

curve 1). A linear relationship between the aggregation rate and the γ chain concentration was observed, at concentrations ranging from 0.75 to 12 $\mu\text{mol/L}$, with complete aggregation observed at 12 $\mu\text{mol/L}$ of γ chain. Both the rate and extent of ADP-induced platelet aggregation were markedly depressed in the presence of purified γ Paris I chains (Fig 3, curve 2).

The effect of purified γ Paris I chain toward platelet aggregation in the presence of normal γ chain was studied by performing mixing experiments. When a mixture of γ Paris I and normal γ chains in a ratio of 3:1 was used, a 30% reduction was observed in the ultimate extent of aggregation (Fig 3, curve 3) as compared with a comparable dilution of normal γ chain with buffer alone (Fig 3, curve 4). No difference was detected when a 1:1 ratio was used (not shown).

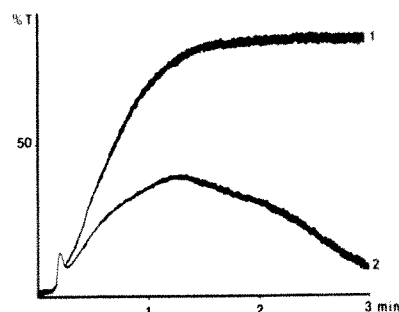


Fig 2. Effect of normal fibrinogen and fibrinogen Paris I on ADP-induced platelet aggregation. Normal (curve 1) or Paris I (curve 2) fibrinogen was added to 300 μL of washed platelets prior to ADP stimulation. The final concentrations were: platelets $3 \times 10^8/\text{mL}$, fibrinogen 1.84 $\mu\text{mol/L}$, and ADP 5 $\mu\text{mol/L}$. T, light transmission.

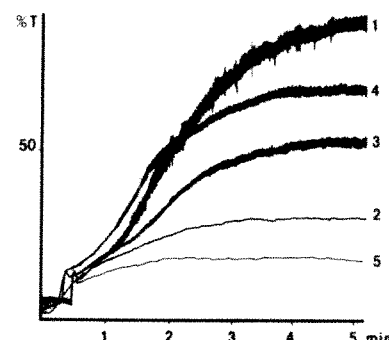


Fig 3. Effect of normal γ chains and γ Paris I chains on ADP-induced platelet aggregation. Purified γ chains were added to 300 μL of washed platelets ($3 \times 10^8/\text{mL}$) prior to ADP (5 $\mu\text{mol/L}$) stimulation. (1) Normal γ chain 4.5 $\mu\text{mol/L}$. (2) γ Paris I chain 4.5 $\mu\text{mol/L}$. (3) Mixture of normal (1.12 $\mu\text{mol/L}$ plus Paris I (3.38 $\mu\text{mol/L}$) γ chains. (4) Normal γ chains 1.12 $\mu\text{mol/L}$. (5) Buffer. T, light transmission.

Isolation and Analysis of CNBr Carboxy-Terminal Peptides From Normal and Paris I γ Chains

The carboxy-terminal CNBr peptide was isolated from both normal and Paris I γ chains digests, as described by Horn.³⁴ Internal fragments containing lactonized homoserine residues (HSe) bound covalently to the resin, whereas the carboxy-terminal CNBr peptide was recovered in the supernatant. Manual Edman degradation of the unbound material, in both cases, identified a Lys-Ile-Ile-Pro amino-terminal sequence, corresponding to residues 385 through 388 in the γ chain (Table 1). The carboxy-terminal residue released on carboxypeptidase A treatment of these two peptides was Val in both cases, as was the carboxy-terminal residue of the intact Paris I or normal γ chain.

On reverse-phase HPLC, the normal and Paris I carboxy-terminal CNBr peptides exhibited an identical retention time. The amino acid composition of the two peptides so purified was very similar (Table 2). Amino-terminal sequence analysis by the manual procedure extended our previous identification for five residues for the normal peptide and for 11 residues in the case of fibrinogen Paris I. Finally, with the use of a gas-phase microsequencer, we obtained the entire amino acid sequence of the 27-residue Paris I peptide. Indeed, it corresponds exactly to the normal sequence of the γ chain extending from residue 385 to the carboxy-terminal Val 411 (Table 1). Three additional degradation cycles gave no evidence for any liberated PTH amino acids beyond Val 411.

DISCUSSION

Human fibrinogen γ chain is involved in several functions, all of which require the participation of its carboxy-terminal region. A complementary polymerization site is located within segment 374 through 396⁴³ and the cross-linking sites between positions 398 and 406.⁴⁴ The 397 through 405 segment participates in the interaction with the staphylococcal clumping receptor,^{19,45} whereas the continuous sequence consisting of the ultimate carboxy-terminal amino acids 400 through 411 of the γ chain constitutes the platelet membrane recognition site.¹⁶ Fibrinogen Paris I is characterized by the

Table 1. Carboxy-Terminal CNBr Peptides of Fibrinogen Gamma Chain: Amino Acid Sequence

	385	390					395				400				405				410									
Theoretical	K	I	I	P	F	N	R	L	T	I	G	E	G	Q	H	H	L	G	G	A	K	Q	A	G	D	V		
Normal	1	K	I	I	P	*																				*V		
	2	K	I	I	P	F	*																					
Paris I	1	K	I	I	P	*																				*V		
	2	K	I	I	P	F	N	R	L	T	I	G	*															
	3	K	I	I	P	F	N	R	L	T	I	G	E	G	Q	Q	H	H	L	G	G	A	K	Q	A	G	D	V*

1. CNBr peptide unbound to the triethylene-tetramine resin in the procedure of Horn and colleagues.³⁴ The amino-terminal sequence was determined by the manual liquid-phase dimethylaminoazobenzene isothiocyanate phenylisothiocyanate (DABITC/PITC) technique. The carboxy-terminal residue was identified after digestion with carboxypeptidase A.

2. Manual degradation (as above) of the carboxy-terminal CNBr peptide purified by reverse-phase high performance liquid chromatography.

3. Automatic gas-phase degradation of the carboxy-terminal CNBr peptide of the γ Paris I chain purified by HPLC. Thirty degradation cycles were performed. Three additional cycles failed to give evidence for any PTH amino acid beyond Val 411.

In each experiment, sequence was determined up to *

Table 2. Carboxy-Terminal CNBr Peptide of the Fibrinogen γ Chain: Amino Acid Composition

AA	Paris I	Normal	Theoretical
Asx	1.8	2.0	2
Ser	0.2	0.0	0
Thr	0.9	1.3	1
Glx	3.7	3.5	4
Pro	—	—	1
Gly	5.0	4.5	5
Ala	1.9	2.0	2
Val	1.1	1.0	1
Met	0.0	0.0	0
Ile	2.9	2.3	3
Leu	1.8	2.0	2
Tyr	0.3	0.1	0
Phe	1.2	1.0	1
His	1.5	1.8	2
Lys	2.4	2.2	2
Arg	1.1	1.2	1

coexistence of normal and elongated γ chains.^{21,22} This report demonstrates that, in addition to the previously described defects in polymerization,⁴⁶ cross-linking^{21,22} and plasmic degradation,²³ both the rate and extent of ADP-induced platelet aggregation are depressed in the presence of fibrinogen Paris I. Furthermore, the isolated γ Paris I chain is unable to support ADP-induced platelet aggregation, even though its terminal sequence is normal from position 384 and includes residues 397 through 411, which account for this activity in normal γ chains.

The functional defect of the carboxy-terminal part of Paris I γ chain is therefore not related to an abnormality in the primary structure of the region extending from residue 384 through residue 411, and the elongation of the γ Paris I chain is due to an internal insertion of peptidic material upstream from Met 384. As suggested by NaDodSO₄ PAGE,²¹ this insertion consists of ~22 residues. To our knowledge, this

constitutes the largest internal insertion observed to date in human protein mutants. Most of the other elongated variants result either from a frameshift mutation in the vicinity of the termination codon or from a mutation of this codon itself and therefore have an abnormal carboxy-terminal extremity.

The γ Paris I chain may be unable to bind to the platelet receptor because of a conformational change that results in masking of its carboxy-terminal region. Alternatively, the binding of γ Paris I chains to stimulated platelets, although occurring through the normal carboxy-terminal recognition site, may result in ineffective bridging between adjacent platelets. We favor this second possibility because receptor occupancy by elongated and nonfunctional γ chains could account for an inhibitory effect and explain the disaggregation phenomenon that occurs with platelet aggregated with fibrinogen Paris I. Defective binding to platelets has also been described for the extended γ' variant of normal human fibrinogen,⁴⁷ but in this case, deletion of the terminal residues 408 through 411 of the specific platelet recognition site probably accounts for this behavior.⁴⁸

The γ Paris I chain is absent (or present in markedly reduced amounts) from the Paris I subject's platelet fibrinogen.⁴⁹ In addition, the plasma fibrinogen γ' variant is not found in normal platelet fibrinogen.^{35,50} These observations raise the question of whether the ability of the γ chain to be packaged in platelet α -granules is dependent on its ability to bind to a membrane receptor for the γ chain.

In conclusion, study of the reactivity of fibrinogen Paris I and γ Paris I chains in platelet aggregation has provided a unique opportunity to demonstrate that the presence of the γ chain platelet recognition site in the fibrinogen molecule is necessary but not alone sufficient to support normal ADP-induced platelet aggregation. That is, there appears to be an absolute requirement for normal conformation of the γ chain or availability of its carboxy-terminal sequence during the interaction of fibrinogen with platelets.

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CONCISE REPORT

Blood Cells Participate in the Fibrinolytic Response to Tissue-Type Plasminogen Activator

By M. Wahbi Hammouda and Leonard A. Moroz

Exercise and venous occlusion stimulate fibrinolysis. In addition to increased concentrations of tissue-type plasminogen activator (tPA) and increased plasmin-mediated fibrinolysis in plasma, these stimuli produce additional cellular-phase activity in blood that is of the same magnitude as the plasma response. To determine whether tPA alone, rather than other consequences of these stimuli, is responsible for the cellular response, the *in vitro* effects of tPA on whole blood, plasma, and calculated cellular-phase (whole blood minus plasma) activities were determined by solid-phase radiofibrin assay on venous blood from ten normal subjects (seven men, three women). At tPA concentrations encompassing physiological and therapeutic levels (5 to 100 ng/mL; 0.7 to 14 IU/mL), increments in whole blood were consistently in excess of those in com-

panion plasma and represented increased cellular-phase activity equivalent in magnitude to the well-known increase in plasma activity. Fibrinolytic activity produced by 10 to 20 ng tPA/mL (1.4 to 2.8 IU/mL) was consistently detected in whole blood and plasma by 60 minutes, with higher concentrations (100 ng or 14 IU tPA/mL) detectable after a five-minute assay in all subjects. Thus, tPA alone, without invoking fibrinolytic factors extraneous to blood or other effects of exercise or venous occlusion, accounts for both cellular and plasma responses to these stimuli. The considerable cellular response, the mechanism of which remains to be elucidated, may constitute a determinant of individual therapeutic responsiveness to tPA.

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THE GENERATION of the fibrinolytic enzyme plasmin from its plasma proenzyme plasminogen by tissue-type plasminogen activator (tPA) released from vascular endothelium is a major mechanism for physiological or pathological stimulation of fibrinolytic activity in plasma and the basis for the increasing therapeutic use of tPA for thrombolysis.¹ Two well-studied stimuli of its release *in vivo* are venous occlusion and exercise.² In both, the participation of tPA has been documented functionally and immunochemically.²⁻⁶

In addition to the plasminogen-plasmin system¹ and other nonplasmin fibrinolytic enzymes⁷ in the plasma phase of blood, there is accumulating evidence for a quantitatively important cellular-phase component to blood fibrinolytic activity both in normal and in physiologically, pathologically, and pharmacologically altered fibrinolysis.⁸ When methods capable of simultaneously monitoring whole blood and plasma fibrinolytic activities are used, this cellular contribution is demonstrable in the blood of normal individuals⁹; in the fibrinolytic responses to such stimuli as venous occlusion,⁶ venous thromboembolism,¹⁰ exercise,¹¹ surgical trau-

ma,¹² urokinase,¹³ and salicylates^{9,14,15}; and in decreased cellular activity in systemic lupus erythematosus.¹⁰ The blood neutrophil, with its abundant fibrinolytic enzymes, lysosomal elastase, and cathepsin G,¹⁶⁻¹⁸ is a major candidate for a participating cell. It has been directly implicated in the responses to surgical trauma,¹² urokinase,¹³ and salicylates.⁹ However, a role for other blood cells, particularly the monocyte, which has demonstrated fibrinolytic potential,^{19,21} cannot be excluded.

In studies of the fibrinolytic responses to exercise¹¹ and venous occlusion,⁶ we found the expected increases, attributable to tPA, in plasma fibrinolytic activity. With both stimuli, an additional response was measurable in blood that was of the same magnitude as the response in plasma and that could only be explained by the participation of blood cells. This cellular-phase increment did not correlate with blood counts of individual cells, thereby suggesting a functional change in one or more cell types. Such a response might be due to direct or indirect effects of tPA on plasminogen, other plasma proteins and blood cells, or might reflect parallel but unrelated metabolic effects of venous occlusion or exercise on cellular fibrinolysis. To clarify these alternatives, we have examined the effects of tPA on fibrinolytic activities of whole blood and plasma *in vitro*.

MATERIALS AND METHODS

Venous blood samples were obtained in the nonfasting state from ten healthy young adults (seven men, three women), with application of a tourniquet for less than one minute and anticoagulation with heparin (10 U/mL). Fibrinolytic activities of paired whole blood and platelet-poor plasma samples were determined by a ¹²⁵I-labeled fibrin solid-phase radiometric test tube assay,²² as described previously,⁹ with and without the addition of tPA (DNA recombinant tissue-type plasminogen activator rtPA, kindly provided by Genentech, Inc. South San Francisco, CA). The specific activity of this preparation (lot no. H9075BX) was 142,000 IU/mg by calibration with the World Health Organization international reference standard for tPA by fibrin plate assay. The activator was added to blood or plasma in a 0.1 vol of isotonic Tris-NaCl buffer, pH 7.4,⁹ to yield the final concentrations indicated. Fibrinolytic activities were expressed as nanograms of fibrin lysed.

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The rate of increase in individual whole blood and plasma fibrinolytic activities was determined by incubation for periods from 0 to 30 minutes at a tPA concentration of 100 ng/mL. The effects of varying concentrations of tPA (5 to 50 ng/mL) were determined by assay of whole blood and plasma for 60 minutes. In addition to absolute whole blood and plasma activities determined with and without tPA for individual subjects, individual cellular-phase activities were calculated from the difference between paired whole blood and plasma activities, and tPA-induced increments in cellular-phase and plasma activities were calculated and compared.

Statistical analysis using a Statpak program was by Student's *t* test for paired and unpaired data.

RESULTS

The addition of tPA to whole blood or plasma from normal subjects at concentrations of 5 to 50 ng/mL resulted in the expected progressive increase in plasma fibrinolytic activity (Fig 1, open bars), with a tenfold increase in mean plasma activity at a tPA concentration of 50 ng/mL. However, there was a similar but greater increase in whole blood activity (represented in Fig 1 by open bars plus solid bars) that could not be attributed to the increase in plasma activity alone. When this cellular-phase component was estimated from the difference between individual whole blood and plasma values (Fig 1, hatched bars), it was found, on average, not to differ significantly ($P > .1$) from corresponding mean plasma activities at each concentration of tPA tested. There were significant increases in mean whole blood and plasma activities at 10 ng/mL ($P < .05$ and $P < .005$, respectively).

This relationship was maintained when tPA-related increments in plasma and cellular-phase activities were calculated by subtracting buffer control values determined without tPA (Fig 2). Mean individual increments in calculated cellular-phase activity were similar to but not significantly different ($P > .1$) from mean individual increments in plasma activity at each tPA concentration tested.

A cellular-phase response to tPA was also observed when the rate of generation of fibrinolytic activity was determined

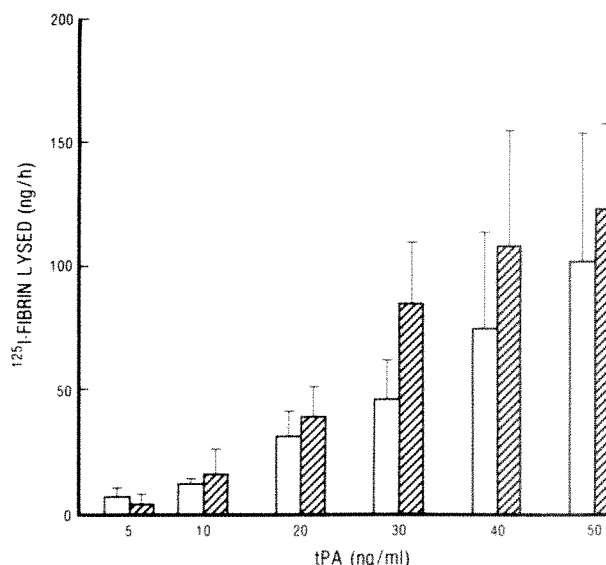
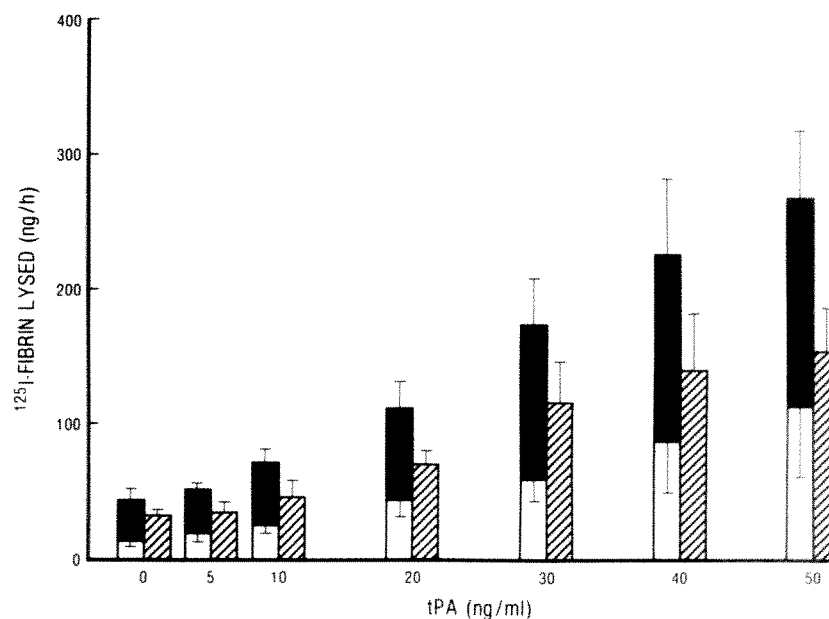


Fig 2. Mean individual increments in plasma (open bars) and calculated cellular-phase activities (hatched bars), with different concentrations of tPA added to normal blood and plasma, calculated from the data shown in Fig 1.

in whole blood and plasma to which a constant concentration of tPA (100 ng/mL) had been added (Fig 3). Again, there was a progressive increase in tPA-stimulated plasma fibrinolytic activity with time (open bars) and a quantitatively greater increase in whole blood activity (open bars plus solid bars), with mean individual increments in calculated cellular-phase activity (hatched bars) that were similar ($P > .1$) to corresponding mean individual increments in plasma activity (open bars) at each time point. Although the mean values for whole blood and calculated cellular-phase activity for the entire group of normal subjects only differed significantly from zero time values after a 15-minute assay ($P < .01$), which reflects the individual variation in responses

Fig 1. Effects of tPA on fibrinolytic activities of whole blood and plasma in vitro. Mean activities of plasma (open bars) for ten normal adults (seven men, three women) are superimposed on corresponding mean whole blood activities (open bars plus solid bars). Mean individual calculated cellular-phase activities (whole blood activity minus plasma activity) are indicated by the hatched bars. Fibrinolytic activities were determined by a solid-phase radio-fibrin assay for 60 minutes at 37°C. Vertical bars denote 1 SEM.



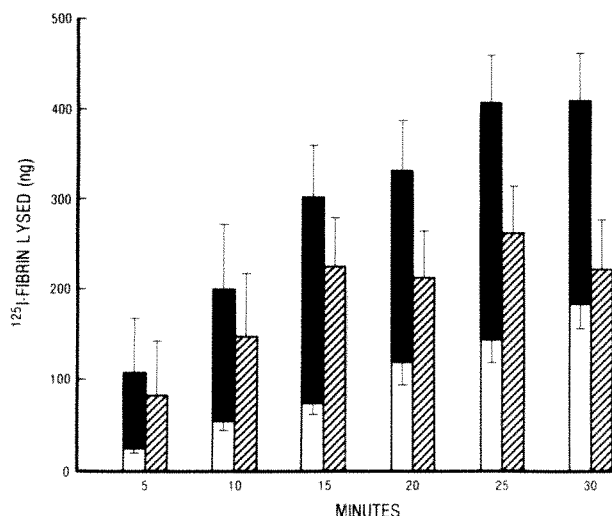


Fig 3. Mean individual increments with time in whole blood (open bars plus solid bars), plasma (open bars), and calculated cellular-phase fibrinolytic activities (hatched bars) due to addition of tPA (100 ng/mL).

to tPA in vitro, there was, for whole blood and plasma of each subject tested, a detectable increase in fibrinolytic activity after assay for only five minutes.

DISCUSSION

This study demonstrates that a purified preparation of tPA added to normal blood in vitro produces a cell-related fibrinolytic response that is in addition to and of the same magnitude as the well-known plasmin-mediated fibrinolytic response in plasma. These findings are relevant to the understanding of the action of tPA and other activators in physiologically and pathologically altered fibrinolysis and to the therapeutic use of tPA including the monitoring of its fibrinolytic effects.

The similarity between the increases in cellular-phase and plasma fibrinolytic activities noted here in vitro reproduce the findings with exercise,¹¹ venous occlusion,⁶ and urokinase administration¹³ in vivo. The fibrinolytic responses to exercise and venous occlusion are associated with increased plasma tPA levels.²⁻⁶ The present in vitro study indicates that the large cellular-phase response to these stimuli is explicable by effects of tPA on blood components alone,

without invoking nonfluid cellular elements such as endothelium or other physiological or metabolic consequences of venous occlusion or exercise distinct from the tPA-plasminogen system and its interactions with other plasma proteins and cells.

The identity of the blood cells and the mechanisms involved remain to be elucidated. The neutrophil is a candidate because of its demonstrated participation in blood fibrinolysis in other contexts⁸ including its partial explanation of the cellular responses to urokinase.¹³ However, other blood cells such as monocytes¹⁹⁻²¹ have fibrinolytic potential,⁸ although their contribution to physiological fibrinolysis in blood is uncertain. Dissection of the tPA effect will require a systematic study of interactions among tPA, plasminogen (and other plasma proteins), and purified preparations of individual blood cell types since the effect is not reproduced by simple addition of preformed plasmin to blood cells.¹³

There is need for a rapid, sensitive, and simple method of monitoring the fibrinolytic effects of tPA and other plasminogen activators in clinical practice,²³ particularly since optimal therapeutic dosages for plasminogen activators remain to be established. The radiometric test tube assay used in this study meets these requirements. In studies of coronary thrombolysis with various dosages of recombinant tPA preparations very similar to the one used here, mean peak values from 1,200 to 3,300 ng tPA/mL have been reported.²⁴⁻²⁶ In the present study, a relatively low concentration from the therapeutic standpoint (100 ng/mL) was detectable in whole blood or plasma after only five minutes of assay (Fig 3), and even lower levels were measurable within acceptable assay times (10 to 20 ng/mL) in one hour (Fig 1), which indicates the applicability of the radiometric assay to the therapeutically relevant range of tPA concentrations. The method has the additional advantages, when applied to whole blood, of eliminating the time required for centrifugal preparation of plasma and permitting the detection and quantitation of the cellular component of the fibrinolytic response to tPA and other activators. This component, which is considerable, may constitute a variable determining individual responsiveness to tPA and other activators.

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CONCISE REPORT

Deoxycytidine Preferentially Protects Normal Versus Leukemic Myeloid Progenitor Cells From Cytosine Arabinoside-Mediated Cytotoxicity

By Kapil Bhalla, William MacLaughlin, John Cole, Zalmen Arlin, Michael Baker, Gary Graham, and Steven Grant

We examined the ability of high concentrations of the naturally occurring nucleoside deoxycytidine (dCyd) to reverse the cytotoxicity of high (eg, $\geq 10^{-5}$ mol/L) concentrations of 1- β -D arabinofuranosylcytosine (Ara-C) toward normal (CFU-GM) and leukemic myeloid progenitor cells (L-CFU). Leukemic myeloblasts from patients with acute nonlymphocytic leukemia (ANLL) and normal human bone marrow mononuclear cells were cultured in soft agar in the continuous presence of 10^{-5} to 5×10^{-5} mol/L of Ara-C together with dCyd (10^{-4} to 5×10^{-3} mol/L). Administration of 10^{-5} mol/L of Ara-C alone eradicated colony formation in all samples tested. Coadministration of 10^{-3} mol/L of dCyd restored 72.2% of control colony formation for CFU-GM, but only 10.9% for L-CFU. When higher concentrations of Ara-C (eg, 5×10^{-5} mol/L) were administered,

dCyd-mediated protection toward CFU-GM decreased, but remained significantly greater than that observed for L-CFU. Incubation with 10^{-3} mol/L of dCyd reduced the 4-hour intracellular accumulation of the triphosphate derivative of Ara-C (Ara-CTP) in both normal and leukemic cells by >98%; under identical conditions, a significant expansion of the intracellular of the triphosphate derivative of dCyd (dCTP) pools was observed in normal bone marrow mononuclear cells but not in leukemic blasts. This finding was associated with a greater reduction in Ara-C DNA incorporation in normal elements. These in vitro studies suggest that dCyd may preferentially protect normal v leukemic myeloid progenitor cells from the lethal actions of high-dose Ara-C.

THE DEOXYCYTIDINE (dCyd) analog 1- β -D arabinofuranosylcytosine (Ara-C) is an effective antileukemic drug in humans.¹ Both dCyd and Ara-C (and their metabolites) are competitive substrates for a variety of intracellular enzymes including dCyd kinase,² cytidine deaminase,³ and dCMP deaminase.⁴ In addition, dCyd and Ara-C are ultimately converted to their triphosphate derivatives (dCTP and Ara-CTP), which compete for binding to the enzyme DNA polymerase⁵ and for incorporation into DNA.⁶ The latter process has recently been highly correlated with Ara-C-mediated lethality toward leukemic cells in vitro.⁶ Because of its ability to antagonize Ara-C metabolism at various levels, dCyd has been associated with reversal of Ara-C toxicity both in vitro⁷ and in vivo.⁸

Intermittent high-dose infusions of Ara-C have achieved responses in up to 50% of patients with acute nonlymphocytic leukemia (ANLL) in relapse.⁹ More recently, high-dose Ara-C was administered as a continuous infusion for 96

hours to patients with refractory ANLL, and steady-state plasma levels in excess of 10^{-5} mol/L were achieved.¹⁰ Although significant oncolytic responses have been observed, the dose-limiting toxicity has been myelosuppression.^{10,11} Studies in intact animals showed that dCyd can ameliorate the host toxicity of otherwise lethal doses of Ara-C, leading to a net gain in therapeutic index for the combination.¹² More recently, our group reported that dCyd (10^{-4} to 10^{-3} mol/L) selectively enhances the in vitro growth of normal CFU-GM, but not leukemic myeloid progenitor cells (L-CFU).¹³ These studies raise the possibility that high concentrations of dCyd may preferentially protect normal marrow elements from Ara-C-induced lethality, thereby improving antileukemic efficacy. The aim of the present study was to compare the ability of high concentrations of dCyd to reverse the growth inhibitory effects of continuously administered high-dose Ara-C toward normal v L-CFU in culture. An additional aim was to establish the biochemical basis for potential selectivity, including examination of the intracellular accumulation of Ara-CTP and dCTP and the incorporation of Ara-C into DNA of normal and L-CFU.

MATERIALS AND METHODS

Drugs and chemicals. Deoxycytidine and Ara-C were purchased from Sigma Chemicals (St Louis). Drugs were stored as dry powders at -20°C and reconstituted in sterile media prior to use. [^3H] Ara-C (26 Ci/mmol) and [^3H] dCyd (16 Ci/mmol) were purchased from Amersham Radiochemicals (Arlington Heights, IL).

Culture of normal human bone marrow granulocyte macrophage progenitors. Human bone marrow myeloid progenitors were cultured by a previously described double-layer soft agar cloning method.¹⁴ Samples were obtained with informed consent from patients undergoing routine diagnostic bone marrow aspirations for non-neoplastic hematologic disorders that did not involve the myeloid series or from normal volunteers. These studies were sanctioned by the Investigational Review Board of Columbia University. Mononuclear cells were isolated by the method of Boyum,¹⁵ washed twice, and suspended in a mixture consisting of McCoy's 5a medium, 20% fetal calf serum (FCS), and 0.3% Bacto agar (Difco,

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Detroit). Aliquots (0.5 mL) of the mixture were pipetted into 18-mm 12-well plates (Costar, Cambridge, MA) containing a bottom layer consisting of McCoy's 5a medium, 20% FCS, and 0.5% agar. To each well, which contained 10^5 cells/well, was added 0.1 mL of GCT-conditioned medium (GIBCO, Grand Island, NY) as a source of colony-stimulating factor, along with the designated concentrations of dCyd and Ara-C. After the agar was allowed to harden, the plates were placed in a fully humidified, 37°C, 5% CO₂ incubator (Napco, Portland, OR) for 7 days. At the end of this incubation period, colonies, consisting of groups of ≥ 50 cells with granulocytic or macrophagelike appearance, were scored with an Olympus model CK inverted microscope. The effect of Ara-C, (with or without dCyd) on CFU-GM growth was expressed as the percentage of colony formation by drug-treated cells relative to untreated controls.

Culture of leukemic blast progenitors. Leukemic blasts were cultured by a minor modification of previously described technique.¹⁶ T lymphocyte-depleted mononuclear cells were obtained from bone marrow or peripheral blood samples from patients with acute nonlymphocytic leukemia,¹⁷ and plated at 2×10^5 cells/mL, using the bilayer soft agar method previously described for normal CFU-GM. All samples used were comprised of at least 80% blasts. Phytohemagglutinin (PHA) leukocyte conditioned medium (10% vol/vol) was used as a source of colony-stimulating activity.¹⁷ Plates were incubated in a 37°C, 5% CO₂ fully humidified incubator for 7 days, after which colonies, consisting of ≥ 20 blastlike cells, were scored with an inverted microscope. The effect of Ara-C $\pm$ dCyd on L-CFU formation was evaluated in the same manner used for CFU-GM.

Intracellular Ara-CTP and dCTP levels. Normal bone marrow mononuclear cells and leukemic blasts were isolated as described above and suspended in McCoy's 5a medium containing 10% FCS at a cell density of 10^6 cells/mL. The suspension was placed in 25-cm² tissue culture flasks (Corning, Corning, NY) to which were added the indicated concentrations of Ara-C and dCyd. The flasks were placed in the 37°C, 5% CO₂ incubator for 4 hours, after which the suspension was centrifuged at 400g for 8 minutes at 4°C; neutralized HClO₄ soluble extracts of the cell pellet were obtained as described previously.¹⁴ Immediately prior to analysis, the acid-soluble extracts were treated by periodate oxidation¹⁸ to remove endogenous ribonucleotides, and Ara-CTP and dCTP were quantitated by a high-pressure liquid chromatography method¹⁹ using Waters HPLC system including a 440 dual-wavelength absorbance detector (Waters, Milford, MA). In brief, a Radial Pak SAX anion exchange column (Waters) was used with a 300 mmol/L NH₄H₂PO₄ buffer (pH 3.5) plus 2% methanol as the mobile phase at a 2.5 mL/min flow rate. Separation of the two metabolic products was >1.5 minutes. Values were expressed as pmol/ 10^6 cells $\pm$ SEM.

Ara-C incorporation into nucleic acids. After cells were isolated, washed, and suspended as described above, they were placed in T-flasks containing 10^{-5} mol/L of [³H] Ara-C $\pm$ 10^{-3} mol/L of dCyd. The flasks were then incubated at 37°C for 4 hours, after which the suspension was transferred to 50-mL tubes and centrifuged at 400 g for 8 minutes at 4°C. The cell pellet was washed twice with cold phosphate-buffered saline (PBS) to remove the radiolabeled drug. DNA was isolated and purified by pronase digestion, phenol extraction, and ethanol precipitation as previously described.²⁰ After centrifugation at 9,000 g at 4°C for 30 minutes, the DNA was resuspended in Tris-HCl buffer, quantitated spectrophotometrically, and aliquots were removed and placed in scintillation vials to determine the radioactivity. Values for each condition are expressed as pmol of Ara-C/ μ g of DNA.

Statistical analysis. Significant differences between experiment groups were assessed using Student's *t* test for paired observations.

RESULTS

The effect of dCyd on the growth inhibitory effects of continuously administered high-dose Ara-C (10^{-5} or 5×10^{-5} mol/L) toward normal and leukemic myeloid progenitors is shown in Table 1. Exposure of cells to Ara-C alone resulted in complete inhibition of colony formation in all samples (not shown). Coadministration of 10^{-3} mol/L of dCyd together with 10^{-5} mol/L of Ara-C restored $72.2\% \pm 9.1\%$ of control CFU-GM colony formation. In contrast, under identical conditions, L-CFU growth remained substantially inhibited at $10.9\% \pm 3.7\%$ of control ($P < .01$). When CFU-GM were cultured in the presence of 5×10^{-5} mol/L of Ara-C in conjunction with a hundred-fold excess of dCyd (5×10^{-3} mol/L), the degree of protection declined (eg, $42.4\% \pm 5.0\%$ of control). Restoration of colony formation remained significantly greater than that observed for L-CFU under the same conditions, however ($7.8\% \pm 3.0\%$ of control). Culture of cells with dCyd in excess by only tenfold resulted in very little protection of either normal or leukemic cells from Ara-C-mediated toxicity.

The effect of dCyd on the 4-hour intracellular accumulation of Ara-CTP in normal bone marrow mononuclear cells and leukemic myeloblasts is shown in Table 2. With 10^{-5} mol/L of Ara-C alone, the intracellular accumulation of Ara-CTP was not significantly different for normal and leukemic cells (eg, 33.32 ± 10.40 v 28.18 ± 8.28 ; $P > .10$). Under these conditions, neither were intracellular dCTP

Table 1. Effect of dCyd on the Inhibitory Activity of Ara-C Toward L-CFU and CFU-GM Growth

Condition	Control Colony Growth (%)	
	L-CFU (n = 10)	CFU-GM (n = 22)
Ara-C 10^{-5} mol/L	0	0
Ara-C 10^{-5} mol/L + dCyd 10^{-4} mol/L	12.9 ± 7.0	3.2 ± 1.1
Ara-C 10^{-5} mol/L + dCyd 10^{-3} mol/L	10.9 ± 3.7	$72.2 \pm 9.1^*$
Ara-C 5×10^{-5} mol/L	0	0
Ara-C 5×10^{-5} mol/L + dCyd 10^{-3} mol/L	3.8 ± 2.8	9.4 ± 2.2
Ara-C 5×10^{-5} mol/L + dCyd 5×10^{-3} mol/L	7.8 ± 3.0	$42.4 \pm 5.0^*$

Normal cells and L-CFU were cultured in soft agar in the continuous presence of Ara-C (10^{-5} mol/L and 5×10^{-5} mol/L) with or without dCyd (10^{-4} to 5×10^{-3}). After 7-day incubation, colonies, consisting of ≥ 50 (CFU-GM) or ≥ 20 (L-CFU) cells, were scored with an inverted microscope. Values are the percentage for each condition relative to control colony formation; n, number of samples studied.

*Significantly different ($P < .05$) from L-CFU growth under identical conditions.

Table 2. dCTP and Ara-CTP Accumulation and [³H] Ara-C DNA Incorporation in Normal and Leukemic Bone Marrow Mononuclear Cells

Marrow	Ara-CTP (pmol/10 ⁶ Cells)	dCTP (pmol/10 ⁶ Cells)	[³ H] Ara-C DNA Incorporation (pmol/μg DNA)
Leukemic (n = 5)			
dCyd 10 ⁻⁴ mol/L	—	0.68 ± 0.32	—
dCyd 10 ⁻³ mol/L	—	1.28 ± 0.65	—
Ara-C 10 ⁻⁵ mol/L	33.32 ± 10.40	0.39 ± 0.36	0.148 ± 0.040
Ara-C 10 ⁻⁵ mol/L + dCyd 10 ⁻⁴ mol/L	1.73 ± 0.43	0.65 ± 0.25	0.030 ± 0.011
Ara-C 10 ⁻⁵ mol/L + dCyd 10 ⁻³ mol/L	0.11 ± 0.09	1.13 ± 0.32	0.007 ± 0.002
Normal (n = 6)			
dCyd 10 ⁻⁴ mol/L	—	10.40 ± 1.57	—
dCyd 10 ⁻³ mol/L	—	23.72 ± 2.51	—
Ara-C 10 ⁻⁵ mol/L	28.18 ± 8.28	0.96 ± 0.72	0.064 ± 0.014
Ara-C 10 ⁻⁵ mol/L + dCyd 10 ⁻⁴ mol/L	0.50 ± 0.46	7.17 ± 2.29*	0.008 ± 0.001
Ara-C 10 ⁻⁵ mol/L + dCyd 10 ⁻³ mol/L	0.14 ± 0.13	9.82 ± 2.18*	0.002 ± 0.0004

*Values significantly different ($P < .05$) from those observed from ANLL cells exposed to identical conditions.

levels significantly different in either cell type. Coincubation with 10⁻³ mol/L of dCyd reduced the 4-hour intracellular accumulation of Ara-CTP in both normal and leukemic cells by >98% (0.14 and 0.11 pmol/10⁶ cells, respectively). Under these conditions, however, a significant expansion of intracellular dCTP pools was observed only in normal bone marrow mononuclear cells (0.96 to 9.82 pmol/10⁶ cells); dCTP pools in leukemic cells were expanded to a much smaller degree (0.39 to 1.13 pmol/10⁶ cells).

The effect of dCyd on the 4-hour incorporation of [³H] Ara-C (10⁻⁵ mol/L) into normal and leukemic cell DNA is shown in Table 2. When cells were exposed to 10⁻⁵ mol/L of Ara-C, leukemic blasts incorporated more of Ara-C than did normal bone marrow mononuclear cells (0.148 v 0.064 pmol of Ara-C/μg of DNA, respectively); however, this difference was not statistically significant ($P > .05$). Coadministration of 10⁻³ mol/L of dCyd reduced the Ara-C DNA incorporation to a greater extent in normal v leukemic bone marrow cells (.002 v .007 pmol of Ara-C/μg of DNA). The magnitude of this difference in Ara-C DNA incorporation did not parallel the significantly greater expansion (~eightfold) of dCTP pools in normal marrow cells under identical conditions, however.

DISCUSSION

Pharmacokinetic studies of Ara-C have consistently demonstrated that steady-state plasma levels of $\geq 10^{-5}$ mol/L are achieved during continuous-infusion high-dose Ara-C therapy.¹⁰ These Ara-C levels decrease the variability in DNA inhibition,²¹ saturate the capacity of the leukemic myeloblasts to accumulate Ara-CTP,²² and increase the incorporation of Ara-C into DNA.²³ The present study demonstrates that high concentrations of dCyd (eg, $\geq 10^{-3}$ mol/L) preferentially protect normal v L-CFU from the in vitro cytotoxic effects of high-dose Ara-C (eg, 10⁻⁵ and 5×10^{-5} mol/L). Previous investigators have reported that dCyd is relatively ineffective in reversing the lethal actions of Ara-C toward

murine leukemias in vivo¹² and inefficient in antagonizing Ara-CTP formation in some human leukemic myeloblasts in vitro.²⁴ Moreover, we showed that 10⁻⁴ to 10⁻³ mol/L dCyd administered alone preferentially stimulates the in vitro growth of normal but not L-CFU.¹³ Together, these results suggest that differences in the metabolism of high concentrations of dCyd may exist between normal and leukemic cells, and that these differences may account for the relative inability of dCyd to antagonize high-dose Ara-C-mediated lethality in leukemic elements.

In our studies, 10⁻³ mol/L of dCyd dramatically inhibited the formation of Ara-CTP in both normal and leukemic cells. Despite this antagonism, substantial inhibition of leukemic cell clonogenicity was preserved and in all cases exceeded that observed for normal myeloid progenitors. Following exposure to high concentrations of dCyd and Ara-C, normal bone marrow mononuclear cells exhibited a considerable expansion of dCTP pools, whereas leukemic myeloblasts did not. Expansion of intracellular dCTP pools can antagonize Ara-C metabolism and cytotoxicity through a variety of mechanisms, including inhibition of dCyd kinase,² stimulation of dCMP deaminase,⁴ and inhibition of Ara-C incorporation into DNA.⁶ Data from our studies indicate that higher intracellular ratios of dCTP/Ara-CTP in normal v leukemic cells exposed to high concentrations of Ara-C and dCyd result in greater reduction in Ara-C DNA incorporation in normal cells. Although the magnitude of this reduction in Ara-C DNA incorporation did not parallel the eightfold greater expansion of dCTP pools in normal cells, it might be sufficient to account for the preferential reversal of Ara-C-mediated cytotoxicity in these elements.

Despite enhanced antileukemic effects of high-dose Ara-C in clinical trials, severe myelosuppression has been a dose-limiting toxicity.¹¹ If, as suggested by our findings, high concentrations of dCyd selectively antagonize Ara-C-mediated toxicity toward normal myeloid elements, it is possible that continuous-infusion high-dose Ara-C could be administered with improved therapeutic efficacy.

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CONCISE REPORT

Eosinophils Stimulate Fibroblast DNA Synthesis

By Stephanie H. Pincus, Kashi S. Ramesh, and David J. Wyler

Fibrosis complicates a number of chronic inflammatory diseases and occurs in some conditions following chronic hypereosinophilic syndromes. We assessed whether eosinophils might be a source of fibrogenic factors. Extracts of human and guinea pig cell populations enriched for eosinophils contained substances that stimulated tritiated thymidine incorporation by human fibroblasts. Supernatants

derived from resting eosinophils and extracts prepared from eosinophil granules also contained fibrogenic factors. Our findings demonstrate a new potential role for eosinophils and suggest a causal relationship between tissue eosinophilia and scar formation in certain parasitic conditions.

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FIBROSIS complicates a number of chronic inflammatory diseases. This observation has motivated investigations of the relationship between inflammatory cells and fibroblasts in fibrogenesis. Some soluble products of chronic inflammatory cells stimulate in vitro a variety of fibroblast functions that are considered important factors in scar formation. Mononuclear phagocytes and lymphocytes, when appropriately activated, secrete factors that can promote fibroblast growth as well as synthesis and secretion of collagen, fibronectin, collagenase, and prostaglandins.¹

Tissue fibrosis has also been observed in selected conditions following chronic hypereosinophilic syndromes in which peripheral blood eosinophilia and tissue eosinophilia occur.² However, the relationship between eosinophilia and fibrosis has not been explored. Accordingly, we assessed whether eosinophils, like macrophages and lymphocytes, might be a source of fibrogenic factors. We now report the presence of eosinophil-derived factor(s) that stimulate fibroblast DNA synthesis in vitro. This finding draws attention to a previously unappreciated source of fibroblast stimulating factors that may participate in fibrogenesis. Our findings also suggest an additional potential role for the eosinophil, a cell whose functions are still incompletely understood.

MATERIALS AND METHODS

Preparation of leukocytes. Human eosinophils were purified on discontinuous metrizamide gradients (Accurate Chemical, Westbury, NY) as previously described.³ In brief, heparinized (10 U/mL of preservative-free heparin, A.H. Robbins, Richmond, VA) blood drawn from healthy normal volunteers was mixed with 4.5% Dextran (T500, Pharmacia Laboratories, Piscataway, NJ, or Dextran 150,000, Sigma Chemical, St Louis) in 0.15 mol/L of phosphate-buffered saline (PBS, pH 7.4) and was allowed to sediment for 30 minutes at 37°C. The leukocyte-rich plasma was removed and washed twice with Eagle's minimal essential medium (MEM, GIBCO, Grand Island, NY) containing 100 U/mL of penicillin, 100 µg/mL of streptomycin, 10% heat-inactivated fetal calf serum

(FCS, Flow Laboratories, Rockville, MD) and 2 mmol/L of L-glutamine. The cells were resuspended to 1×10^6 /mL and layered on discontinuous gradients. After centrifugation, the fractions were counted and pooled. Preparations contained >85% eosinophils; 15% were exclusively neutrophils. For comparison studies between eosinophils and neutrophils, neutrophils were harvested from the 23% metrizamide layer (99% neutrophils).

Guinea pig peritoneal exudate eosinophils were harvested from animals receiving 1 mg of polymyxin B intraperitoneally every week, as previously described.⁴ Peritoneal exudates were collected, washed in PBS, and layered on discontinuous metrizamide gradients. Preparations contained >95% eosinophils. Trypan blue dye exclusion revealed >95% viability of both human and guinea pig cells.

Preparation of cell extracts. Leukocytes were washed three times and suspended in Hanks' balanced salt solution (HBSS). Cells were subjected to six freeze-thaw cycles, and the cellular debris was removed by centrifugation at 100 g for 15 minutes. Supernatants were dialyzed against MEM prior to addition to fibroblast cultures. For studies of granular contents, eosinophils suspended at 5×10^6 /mL were homogenized in 0.34 mol/L of sucrose. Cellular debris was removed by centrifugation at 1,000 g for 10 minutes. The granules were then pelleted by centrifugation, resuspended in PBS, and ruptured by repeat freeze-thaw cycles. Experiments using cell-free supernatants were prepared from resting eosinophils, as previously described.³ In brief, eosinophils suspended in HBSS to a concentration of 2×10^6 /mL were incubated for 1 hour at 37°C. Cells were removed by centrifugation, and the supernatants were dialyzed at 4°C overnight against RPMI 1640 medium (nominal exclusion 1,000 kd). Cell viability was >98% by trypan blue dye exclusion.

Fibroblast cultures and proliferation assay. Human fibroblast cultures were obtained from newborn foreskins and processed by our previously published methods.⁵ Confluent cultures were passaged following treatment with trypsin and EDTA. Fibroblasts were used in assays between passages 4 and 12 according to our published methods.⁵ In brief, cells suspended in RPMI 1640 medium supplemented with antibiotics and 10% heated FCS were plated in 24-well polystyrene plates (Costar, Cambridge, MA) at a density of 5×10^4 /well and incubated overnight at 37°C. Cells were washed, and cultures were replenished with serum-free medium. Twenty-four hours later, stimuli were added for an additional 24 hours, the last four with 1.0 µCi/well (final vol 1.0 mL) of [³H]thymidine (specific activity 6.7 Ci/mmol, New England Nuclear). Cells were harvested on glass fiber filters and counted in a scintillation counter in the presence of scintillation cocktail. Means $\pm$ SEM of triplicate samples were determined. Data are expressed as the percentage of the untreated control to minimize variation due to differences in the control recorded in different experiments.

RESULTS

Cell-free extracts of human and guinea pig cell populations highly enriched for eosinophils were prepared by

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freeze-thaw and centrifugation and contained substances which stimulated [^{3} H]thymidine incorporation by human fibroblasts (Fig 1A). Fibroblast response was dose dependent. Maximum stimulation was observed with dilutions of 1:5 of human eosinophil extract and 1:10 of guinea pig eosinophil extract. We also observed that eosinophils can spontaneously release fibroblast-stimulating activity. Supernatants derived from resting eosinophils stimulated fibroblast proliferation (Fig 1B).

In related studies, we prepared extracts of isolated eosinophil and neutrophil granules and assessed their effects on human fibroblasts (Fig 1C). Eosinophil granule extracts stimulated fibroblast [^{3} H]thymidine uptake at all concentrations tested. In contrast, neutrophil granule extracts were inhibitory to fibroblasts. In the presence of neutrophil granule extracts, the magnitude of [^{3} H]thymidine incorporation was below that observed in fibroblasts grown in medium alone. This inhibitory effect was not observed with more dilute (1:500) extracts, and no significant stimulation was observed.

DISCUSSION

Our results indicate that eosinophils contain factor(s) that can stimulate fibroblast [^{3} H]thymidine incorporation which, under the conditions we use, is a measure of fibroblast DNA synthesis and proliferation.⁵ Fibroblast-stimulating activity may be specifically localized to the granule compartment (Fig 1C). This does not exclude the possibility that cytoplasmic fibroblast-stimulating factors may also exist. Nonetheless, that isolated neutrophil-derived granules lack this activity indicates a distinct difference between these granulocyte populations. Furthermore, our ability to detect fibroblast stimulating activity in supernatants of apparently viable eosinophils (Fig 1B) suggests that these cells may spontaneously elaborate low levels of such factors, possibly by degranulation. Promotion of eosinophil degranulation by physiological agents such as interleukin 1⁶ or antibody-mediated adherence to helminths⁷ may also result in elaboration of the fibroblast-stimulating factor(s).

Fibrosis is a frequent complication of certain tissue helminthic infections in which direct interaction of eosinophils and parasites occurs. In schistosomiasis, eosinophils can participate in killing of an infective larval stage⁷ and are also present in large numbers in the granulomas that surround the eggs deposited in tissue. These granulomas secrete fibroblast-stimulating factors that are believed to be responsible for subsequent hepatic fibrosis.⁵ The findings we report here suggest that eosinophils may be a source of the granuloma-derived factor. In lymphatic filariasis, lymphatic scarring occurs following infiltration of eosinophils around the parasites. On the basis of our findings, we propose that one potential mechanism of lymphatic scarring is release of eosinophil-derived fibroblast-stimulating factor(s). Another pathologic condition in which eosinophils may play a role in fibrosis is endomyocardial fibrosis,⁸ a model for which we have previously described in polymyxin B-treated guinea pigs.⁹

We have not yet characterized the eosinophil-derived

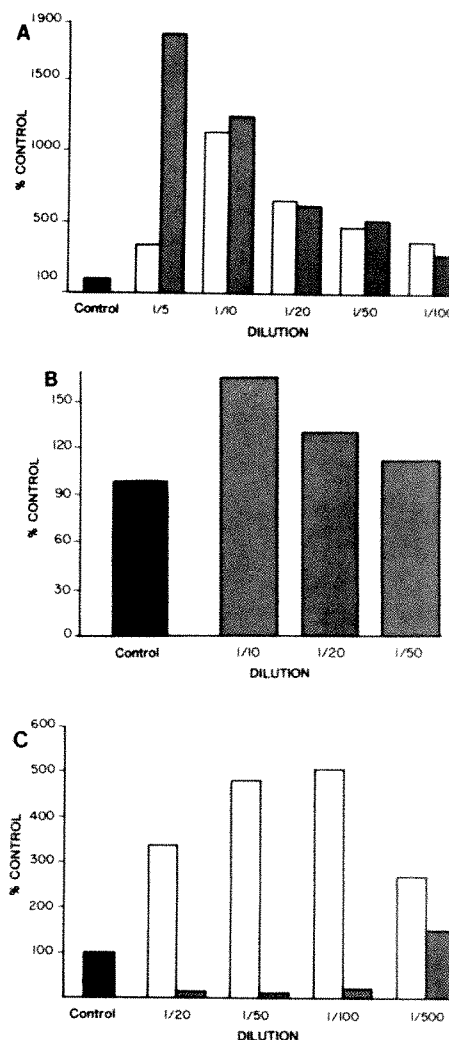


Fig 1. [^{3} H]Thymidine incorporation by human fibroblasts. (A) Effect of human (stippled bar) and guinea pigs (open bars) eosinophil extracts. Freeze-thaw extracts of human or guinea pig eosinophils were prepared as described in the Materials and Methods section, and the incorporation of [^{3} H]thymidine was measured in the indicated dilution and in medium alone. Results, expressed as a percentage of the medium control, are the mean of two experiments performed in triplicate or quadruplicate. The media controls were 732 and 96 cpm for the experiment using human eosinophils and 98 and 96 cpm for the experiments using guinea pig eosinophils. $P \leq .05$ for the significance of the difference between the presence and absence of eosinophils (guinea pig or human) at dilutions of 1:5, 1:10, 1:20, and 1:50. Differences between human and guinea pig eosinophils were significant only at a 1:5 dilution. (B) Effect of supernatant from resting eosinophils. Results are expressed as the percentage of the media control (356, 771, and 9,363 cpm) and are the mean of three experiments performed in triplicate. The dilution indicates the final concentration of supernatant prepared from 2×10^6 eosinophils/mL, as described in the Materials and Methods section. $P \leq .05$ for the significance of the difference between control and 1:10 dilution. (C) Effect of extracts of human eosinophil (open bars) and neutrophil (solid bars) granules. Results are expressed as the percentage of the medium control (918 cpm) and are the mean of a single experiment performed in triplicate. $P \leq .01$ for the significance of the difference between eosinophil granules and neutrophil granules used at final dilutions of 1:10, 1:20, 1:50, and 1:100.

fibrogenic factor(s). Eosinophil granules contain major basic protein, cationic protein, and protein X.¹⁰ Because these factors can be cytotoxic and because we have observed no evidence of toxic effects of eosinophil extracts on fibroblasts, these extracts are unlikely candidates for growth factors.

This report adds eosinophils to the increasing list of cells

that produce fibroblast growth factors. The histopathologic association between tissue eosinophils and subsequent scar formation in certain parasitic infections and various other diseases suggests a causal relationship. Our findings also suggest a new potential role for eosinophils, cells whose functions remain largely ill-defined.

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CONCISE REPORT

Detection of HIV Antigen and Specific Antibodies to HIV Core and Envelope Proteins in Sera of Patients With HIV Infection

By Yunzhen Cao, Fred Valentine, Sally Hojvat, Jean-Pierre Allain, Pablo Rubinstein, Michael Mirabile, Sharon Czelusniak, Michael Leuther, Louis Baker, and Alvin E. Friedman-Kien

The sera of well-characterized populations were examined for three markers of human immunodeficiency virus (HIV) infection: HIV antigen (HIV Ag), and antibodies to HIV envelope (gp41) and core (p24) proteins. Of 563 serum samples tested, 251 were from HIV-infected patients diagnosed as having AIDS manifested by opportunistic infections (AIDS-OI), AIDS-associated Kaposi's sarcoma (AIDS-KS), or AIDS-related complex (ARC). One hundred seventy-six specimens tested were from asymptomatic high-risk individuals, and 136 were from heterosexual control subjects or patients with non-AIDS-related disease. None of the 136 control individuals tested had HIV Ag or HIV antibodies to either p24 or gp41. Of the 427 HIV-seropositive individuals, 99% to 100% were positive for gp41 antibodies to HIV. In contrast, the seroprevalence

of p24 antibodies to HIV varied from 23% to 83% and appeared to be inversely associated with the severity of the patients' clinical symptoms. When specimens were analyzed for the presence of HIV Ag, in seropositive individuals the prevalence rate for this marker was lowest (1.4%) in asymptomatic individuals and highest (50%) in the AIDS-OI diagnosed group. Also, 240 cases with AIDS-KS, AIDS-OI, and ARC and the group of asymptomatic high-risk individuals were analyzed for T helper/T lymphocytes (T4) cell number and T4/T8 ratio; only one (2.0%) HIV Ag-positive case showed a T4 cell number >400 and a normal T4/T8 ratio. These studies appear to demonstrate a direct correlation between the presence of HIV Ag and the severity of clinical complications of HIV infection.

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THE PUTATIVE AIDS human immunodeficiency virus (HIV) has been isolated from peripheral blood mononuclear cells, cerebrospinal fluid, semen, neural tissue, saliva, and tears.¹⁻⁵ Various serologic tests have been developed to detect the presence of antibodies to HIV in patients with AIDS, AIDS-related complex (ARC), and asymptomatic virus carriers; these include radioimmune precipitation, enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and Western blot procedures.

The availability of recombinant HIV proteins has permitted the development of ELISA techniques for the measurement of specific antibodies to HIV core (p24) and envelope (gp41) proteins in the serum or plasma of infected individuals.⁷ In addition, methods have been developed to detect viral antigens in tissue culture fluids using solid-phase, HIV-immune Ig as a capture antibody.⁸ We measured circulating HIV core antigen and antibodies to envelope and core proteins in 563 serum samples, including specimens from patients with AIDS and ARC, and from asymptomatic homosexual individuals at high risk for AIDS, as well as from heterosexual patients with non-AIDS-related diseases and healthy heterosexual control populations.

MATERIALS AND METHODS

Patients and Controls

The sera of 563 individuals, including 251 patients with symptomatic HIV-related disease, 176 asymptomatic high-risk persons, and 136 control subjects were studied. The 251 male patients with HIV-related symptoms included 30 with AIDS manifested by opportunistic infections (AIDS-OI), 142 with AIDS-Kaposi's sarcoma (AIDS-KS), and 79 with ARC.

The population of asymptomatic high-risk individuals examined included 4 homosexual men and 2 heterosexual women who had direct sexual exposure to patients known to have AIDS, 6 intravenous (IV) drug abusers, 16 previously asymptomatic homosexual men who presented with acute herpes zoster infections (VZV), and 148 asymptomatic "healthy" homosexual men.

Included in the control groups were 51 male and female heterosexual patients with illnesses unrelated to HIV; including 14 with systemic lupus erythematosus, 15 who were seropositive for hepatitis

B surface antigen, 12 elderly heterosexual patients with classic KS, 9 heterosexual VZV patients, and 1 undiagnosed healthy HIV-seronegative male patient with unexplained persistent low T helper lymphocyte numbers. Also included as controls were 85 healthy heterosexual laboratory personnel from the New York Medical Center and healthy heterosexual blood donors from the Greater New York Blood Center.

Serum Samples

One-half of the sera samples tested had been frozen and stored at -30°C and thawed just before testing; the remainder of the samples were tested as fresh specimens prior to storage.

HIV Markers

HIV-antigen assay (HTLV-III EIA). The HIV assay evaluated in this study detects primarily the p24 core antigen of HIV as previously described (Abbott Laboratories, N Chicago).⁹

A neutralization procedure was performed on all repeatedly HIV Ag-positive samples to verify the test results. The HIV antigen in the specimen was neutralized by adding 50 µL of a polyclonal human anti-HIV IgG solution prior to running the HIV Ag assay as described above. Both negative and positive control and positive standard controls were mixed with either buffer or neutralizing solution and were run in parallel with the sample specimens; 200 µL of the standard control or sera specimens together with 50 µL of buffer or neutralizing solution were incubated for 2 hours at room temperature. An HIV antibody-coated bead was then added to each sample, and the HIV Ag assay run as previously described. A

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positive result was confirmed when a reduction of at least 50% of the recorded OD was observed in samples incubated with the neutralizing solution as compared with the standard control incubated with the buffer.

HIV anti-core (p24) or anti-envelope (gp41) antibodies. The competitive immunoassay using recombinant antigens (CIA-RA) was described previously (Abbott Laboratories, N Chicago).⁹

Quantitation of T Cell Subsets

OKT4 and OKT8 monoclonal antibodies were purchased from Ortho Pharmaceutical and were used to determine the proportion of positive cells according to the manufacturer's instructions using a FACS 4 instrument.

RESULTS

Prevalence of HIV Antigens and Antibodies to HIV

The prevalence of HIV Ag and of specific antibodies to p24 or gp41 in seropositive members of high-risk groups is shown in Table 1.

None of the healthy heterosexual controls or patients with diseases unrelated to AIDS were positive for any of these markers. From the groups of patients clinically diagnosed as AIDS-OI, AIDS-KS, or ARC, eight samples (3%) were negative for HIV Ag and both HIV antibodies. Thirty-six percent to 50% of the ARC and AIDS patients' samples that were seropositive (or one with both HIV antibodies) showed detectable amounts of HIV Ag. Asymptomatic subjects who were positive for one or both of the HIV antibodies had the lowest prevalence of HIV Ag (1.4%). Most of them, (83.3%), were positive for antibodies to p24.

The serum marker patterns of circulating HIV-Ag and antibodies were remarkably similar in the ARC and AIDS-KS groups. The small cohort of homosexuals with acute VZV infection showed a much higher prevalence of HIV Ag than did the asymptomatic homosexual group and a pattern of HIV markers resembling the AIDS-KS and ARC patients sera tested. In all three groups, HIV Ag was associated with gp41 (86 of 97) and seldom with p24 (10 of 97). This association was statistically significant ($P < .01$). The prevalence of at least one of the HIV markers in the sera of symptomatic and asymptomatic homosexuals was 97% and 49%, respectively.

In all the groups tested, the most consistent marker of HIV infection was the antibody to gp41. The frequency of HIV gp41 antibody in AIDS patients with KS was 99%; in patients with OI it was 100%. In asymptomatic individuals,

80% of those with antibodies to gp41 also had antibodies to p24. The comparable figures for patients with ARC was 51%, for AIDS-KS 50%, and for AIDS-OI only 23%. The prevalence of p24 antibody in patients with AIDS-OI was significantly lower than that of the ARC and AIDS-KS groups and much lower than that of asymptomatic individuals positive for antibody to gp41 ($P < .001$).

Table 2 shows the patterns of HIV markers found in HIV-infected subjects from the various risk groups studied. All three HIV markers, HIV Ag, gp41 antibody, and p24 antibody, occurred together infrequently in all groups. A single ARC patient had only HIV Ag, with no evidence of gp41 or p24 antibodies. In all groups except AIDS-OI, the most prevalent pattern observed was the presence of both HIV p24 and gp41 antibodies, in the absence of HIV Ag. A high percentage of HIV-seropositive individuals had antibody only to gp41 and not to p24. None of the sera contained anti-p24 antibody in the absence of anti-gp41 antibody.

Relationship Between Number of T4(CD4) Cells or T4/T8 Ratio and Presence of HIV Ag, and Antibodies to HIV

Blood from 240 persons was analyzed for the number of T4 lymphocytes and the T4/T8 ratio. In 63 of these, the blood was negative for both antibodies to HIV and for HIV Ag. Data in Table 3 show that among 177 seropositive persons, only one in whom HIV Ag was detected had >400 T4 cells/ μ L or a ratio of T4/T8 >1 . In the remaining 49 HIV Ag-positive individuals, the T4 number was $<400/\mu$ L ($P < .001$).

DISCUSSION

Diagnosis of HIV infection presently relies on either the detection of specific IgG antibodies by various methods or on the isolation of the virus from cultured lymphocytes. Current methods of culturing HIV involve the cocultivation of stimulated patients' cells with normal lymphocytes in vitro. This method may lead to the activation of integrated or otherwise latent HIV. As a consequence, current techniques of culturing HIV may not distinguish between infections in which HIV is actively replicating and those in which the virus is latent. The ability to detect circulating HIV Ag in sera, as demonstrated in this study, may circumvent this problem in many patients, since it is likely that the presence of detectable circulating viral antigen in the serum requires replicating virus.¹⁰ In addition, correlation of the clinical stages of

Table 1. Prevalence of HIV Infection and of HIV Markers in High-Risk Groups and Controls

Diagnosis	No. of Subjects	No. Seropositive (%)	Prevalence (%) of Marker in HIV Seropositives		
			HIV Ag	gp41 Ab	p24 Ab
AIDS-OI	30	30 (100)	50.0	100	23.3
AIDS-KS	142	141 (99.3)	39.7	100	49.6
ARC	79	72 (91.1)	36.0	98.6	51.4
Exposed subjects	12	10 (83.3)	30.0	100	60
H2V homosexual	16	15 (93.8)	26.6	100	60
Asymptomatic homosexual	148	72 (48.6)	1.4	100	83.3
Control/unrelated disease	51	0	0	0	0
Healthy control	85	0	0	0	0

Table 2. Patterns of HIV Markers and T4 Cell Numbers in HIV-Infected Subjects From Various Risk Groups

Clinical Condition	No. of Subjects	No. Seronegative	Patterns of HIV Markers as a Percentage of Seropositive: No. of Patients (%) ($\pm$ HIV Ag, $\pm$ gp41 Ab, $\pm$ p24 Ab)					
			+++	++-	+--	-++	--+	---
AIDS-OI	30	0	3(10)	12(40)	0	4(13.3)	11(36.7)	0
AIDS-KS	142	1	5(3.5)	41(36.2)	0	65(46.1)	20(14.2)	0
ARC	79	7	2(2.8)	23(31.9)	1(1.4)	35(48.6)	11(15.3)	0
Exposed, asymptomatic subjects	12	2	2(20)	1(10)	0	4(40)	3(30)	0
HZV homosexual	16	1	0	4(26.7)	0	9(60)	2(13.3)	0
Asymptomatic homosexuals	148	76	0	1(1.4)	0	60(83.3)	11(15.3)	0
Immunological parameters								
T4 400	50	0	0	1(2.0)	0	48(86)	6(12)	0
T4 400	127	0	7(5.5)	42(33.1)	0	60(47.2)	18(14.2)	0
T4/T8 1	21	0	0	1(4.8)	0	15(74.4)	5(23.8)	0
T4/T8 1	146	0	7(4.5)	42(26.9)	0	88(56.4)	19(12.2)	0

the infections with circulating HIV core antigen and with antibodies against core and envelope proteins may provide further insight into the immunobiology of this disease.

We examined 563 sera by means of ELISA techniques for circulating core antigen and antibodies against gp41 and p24. The data demonstrate that the prevalence of circulating antigen increased with increasing severity of the clinical syndromes associated with HIV. This observation is consistent with the idea that increasing amounts of viral expression occur in the patient as the disease progresses, despite a fall in the number of lymphocytes expressing CD4 (T4 cells), the cell in which most viral replication is believed to occur. The increasing frequency of viral antigen expression as the disease progresses could result either from the production of large amounts of p24 by the remaining CD4 cells or from other types of cells known to support the replication of HIV.

In our series, only one patient (with ARC) had circulating antigen but no detectable antibody to HIV.* Asymptomatic individuals have been described from whom HIV was isolated even though they were seronegative.¹⁰ This observation is most easily explained by assuming that viremia occurs at least transiently after infection, prior to the development of detectable antibodies. In studies in which sequential samples of serum were examined, the presence of HIV Ag in high-risk populations was demonstrated prior to the development of IgG antibodies to HIV.^{7,9} A transient antigenemia may occur in some individuals soon after infection, and the presence of persistent circulating antigen may be associated with symptomatic disease.

The pattern of antigen and antibodies in otherwise asymptomatic homosexual men who have herpes zoster are similar to those seen in patients with ARC, suggesting a biological similarity between these two modes of clinical presentation of HIV infection.¹¹

The prevalence of antibodies to gp41, coded by the *env* gene and antibodies to the core protein p24 also differ in patients with different stages of infection with HIV. All

asymptomatic and symptomatic seropositive individuals had antibody to gp41. No persons in this study were detected with antibody to p24 but without antibody to gp41. The data presented do not enable us to determine whether this reflects the nature of the population groups studied or whether the ELISA test used is more sensitive in detecting antibodies against gp41 than p24, as has been suggested by a comparison of this method with immunoblots.⁷ The prevalence, in seropositive individuals, of antibodies to the core protein p24 decreased from 80% in asymptomatic individuals to 50% in patients with ARC or AIDS-KS and to 23% in patients with AIDS-OI. A sequential loss of detectable antibody to p24 with the onset of symptoms has previously been reported in one adult and one child.¹²

Several possibilities exist that might explain this decrease in antibodies to p24 in patients with more advanced disease. Theoretically, individuals who make antibodies to HIV core as well as envelope proteins after infection may be less likely to develop progressive, symptomatic disease. The step-wise decrease in prevalence of this HIV antibody in patients with AIDS-KS and patients with AIDS-OI, coupled with the frequent development of OI by patients with AIDS-KS makes this explanation less attractive. The ability to make detectable amounts of antibody against p24 may decline as the disease progresses.

In addition, the increased frequency of circulating HIV core protein may, in part, interfere with the ability to detect anti-p24 antibody in patients with more advanced disease, as has been suggested.¹² It is evident, however, from the patterns of antigen and the two antibodies shown in Table 2, that in some specimens both core antigen and antibody to p24 were detected.

The finding that circulating HIV core protein correlates with the severity of the clinical stage of disease resulting from HIV infection is supported by the fact that the presence of core antigen also correlates with the severity of the host's immunologic defect as assessed by depressed numbers of T4 cells (Table 2). In these patients, the low number of T4 cells in turn is correlated with symptomatic disease, as would be expected. The data on T4 cells also confirm that in the groups of patients studied circulating p24 core antigen was present most frequently in individuals with low numbers of circulating T4 lymphocytes. Although HIV preferentially

*The serum sample was repeatedly negative for antibody to HIV; however, a serum sample drawn on the same patient 3 days later contained antibody to gp41 by the same ELISA assay and also contained p24 antigen.

binds to and infects cells bearing the T4 (CD4) surface marker, the increased presence of core antigen in patients with low numbers of T4 lymphocytes in the blood suggests that the expression of HIV infection may be occurring in other types of cells or in T4 lymphocytes that are not circulating.

The data indicate that the development of p24 antigenemia is associated with a loss of antibodies against p24, a low

number of T4 lymphocytes, symptomatic disease, and a poor prognosis.

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CONCISE REPORT

Identification of Normal Human Peripheral Blood Monocytes and Liver as Sites of Synthesis of Coagulation Factor XIII a-Chain

By Laurie J. Weisberg, Donny T. Shiu, Paul R. Conkling, and Marc A. Shuman

Factor XIII is the fibrin-stabilizing factor that covalently cross-links fibrin monomers to form a highly organized, stable fibrin clot. The plasma form of factor XIII is a heterodimer, $\alpha_2\beta_2$, consisting of two α -chains and two β -chains; the intracellular form, such as in platelets and placenta, is a dimer, α_2 , consisting of α -chains only. The catalytic function of factor XIII, a transglutaminase, resides in the α -chain. To address questions regarding sites of synthesis of factor XIII α -chain, an *EcoRI* restriction fragment from the protein-coding region of the factor XIII α -chain cDNA was used as a probe for Northern blot

analysis. The cDNA probe showed hybridization with a single ~4.0-kilobase (kb) message in poly (A)⁺ mRNA prepared from normal human peripheral blood monocytes and normal human liver. The results demonstrate conclusively that factor XIII α -chains are actively synthesized in circulating monocytes and in liver. To our knowledge, these data represent the first demonstration of synthesis of any blood coagulation factor in primary uncultured and unstimulated monocytes or macrophage cells.

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PLASMA FACTOR XIII, the blood coagulation factor that covalently cross-links fibrin monomers into a stable fibrin clot, is a tetrameric complex ($\alpha_2\beta_2$), consisting of two α -chains and two β -chains. The catalytic function as a transglutaminase resides in the α -chain¹ (mol wt ~75,000).² Factor XIII α -chains, but not β -chains, are present intracellularly as dimers (α_2) in human platelets,¹ megakaryocytes,³ placenta and uterus,⁴ and monocytes and macrophages.⁵⁻⁷

Although factor XIII α -chain protein has been identified in monocytes and macrophages,⁵⁻⁷ endocytosis of the factor by these phagocytic cells, rather than direct synthesis, is difficult to exclude. In addition, it would be difficult to determine whether the factor XIII present in monocytes is residual protein that might have been synthesized by monocyte precursors in the bone marrow rather than protein actively synthesized in circulating, mature monocytes. The presence of factor XIII α -chain protein in liver has been controversial, having been found in the human hepatoma cell line Hep G2⁸ and in human⁹ and rabbit¹⁰ hepatocytes by some investigators, but not by others.^{11,12} Grundmann and colleagues¹³ reported that their factor XIII cDNA probe hybridized to placental poly (A)⁺ RNA, but they could not detect a signal for hybridization to liver poly (A)⁺ RNA.

We recently cloned a portion of the factor XIII α -chain cDNA and used part of this cDNA clone as a probe to demonstrate synthesis of mRNA for factor XIII α -chain in the human monocytelike cell line U937.¹⁴ The purpose of the present study was to determine whether normal circulating monocytes and normal human liver tissue actively synthesize factor XIII α -chains.

MATERIALS AND METHODS

Monocyte isolation. Human peripheral blood monocytes were isolated from a normal donor platelet pheresis residue bag. Mononuclear cells were separated on Ficoll-Hypaque gradients¹⁵ (Ficoll-Paque, Pharmacia, Piscataway, NJ) and washed in phosphate-buffered saline (PBS) + 10 mmol/L of EDTA. Monocytes were separated from lymphocytes by discontinuous Percoll (Pharmacia) density gradient sedimentation,¹⁶ and again washed in PBS + 10 mmol/L of EDTA. The monocytes were incubated twice in pooled human serum + 10 mmol/L of EDTA at 37°C for 15 minutes to remove platelets. After further washing in PBS + 10 mmol/L of EDTA, Wright's stain and esterase stain showed 96% monocytes, 4% lymphocytes, and no visible platelets. The cells were washed in

45% Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) 40% heat-inactivated fetal bovine serum, and 15% dimethylsulfoxide. They were slowly frozen in this medium and stored at < -100°C in liquid nitrogen vapor. Immediately before RNA preparation, the monocytes were thawed by incubation in a 37°C water bath for 2 minutes, and were washed three times in prewarmed PBS.

Tissue preparation. Fresh normal human liver, obtained at the time of an open liver biopsy procedure performed for other reasons, was homogenized in the presence of 5 mol/L of guanidine monothiocyanate lysis buffer¹⁷ in a Polytron blender. Frozen placental tissue was similarly homogenized in a Polytron blender; no thawing took place before guanidine monothiocyanate was added to the specimen. Monocytes, after thawing as described above, were homogenized by vigorous vortexing in the presence of guanidine monothiocyanate.

RNA preparation. The guanidine monothiocyanate/tissue homogenates were precipitated in 4 mol/L of LiCl, and RNA was prepared according to the method of Cathala and colleagues.¹⁷ The poly (A)⁺ mRNA fraction was selected by elution from an oligo (dT)-cellulose column.¹⁸

Northern blot analysis. Poly (A)⁺ mRNA was electrophoresed on a 1% agarose 2.2 mol/L formaldehyde gel and blotted to nitrocellulose. The filters were hybridized overnight at 42°C in 50% formamide, 10% dextran sulphate, 1 × Denhardt's solution, 3 × SSC, 50 mmol/L of NaH₂PO₄, 200 µg/mL of sheared salmon sperm DNA, 150 µg/mL of yeast RNA. The molecular probe was a 630-base pair (bp) *EcoRI* restriction fragment of the cDNA for factor XIII α -chain¹⁴ (Fig 1), labeled with [α -³²P]dCTP (Amersham, Arlington Heights, IL) by calf thymus DNA priming. Following hybridization, the filters were rinsed once at room temperature in 2 × SSC, and then washed twice at 50°C in 0.1 × SSC, 0.1% sodium dodecyl sulfate (SDS). The hybridized filters were subjected to autoradiography at -70°C using Kodak XAR-2 film and DuPont Cronex intensifying screens.

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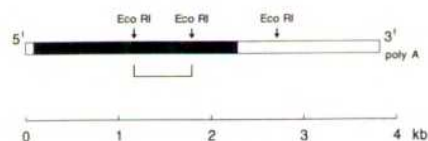


Fig 1. Factor XIII a-chain cDNA.¹⁹ Bracket: Location of the *Eco*RI restriction fragment ³²P-radiolabeled as a probe for hybridization in Northern blot analyses. Solid bar: Location of the protein-coding sequence for factor XIII a-chain.

RESULTS

In the first experiment, RNA was isolated from human peripheral blood monocytes and, as a positive control, from frozen human placental tissue. One of our *Eco*RI restriction fragments from the cDNA for factor XIII, previously described,¹⁴ was 630 bp long (Fig 1) and was located within the protein-coding region.¹⁹ This fragment was ³²P-radiolabeled and used as a probe for Northern blot analysis of the monocyte and placental RNA samples. The autoradiogram (Fig 2) shows that there is a single ~4.0-kb message for factor XIII a-chain in human peripheral blood monocytes and placenta. Poly (A)⁺ mRNA from K562 cells (human chronic myelogenous leukemia, ATCC CCL 243) showed no hybridization. K562 cells were chosen because they express platelet glycoproteins,²⁰ suggesting that they might synthesize factor XIII a-chain, another platelet protein.

In the second experiment, the same ³²P-radiolabeled probe was hybridized to poly (A)⁺ mRNA from fresh human liver, again using placental poly (A)⁺ mRNA as a positive control and K562 cell poly (A)⁺ mRNA as a negative control for Northern blot analysis. Autoradiography (Fig 3) demonstrates the presence of a single ~4.0-kb message for factor XIII a-chain in human liver and placenta.

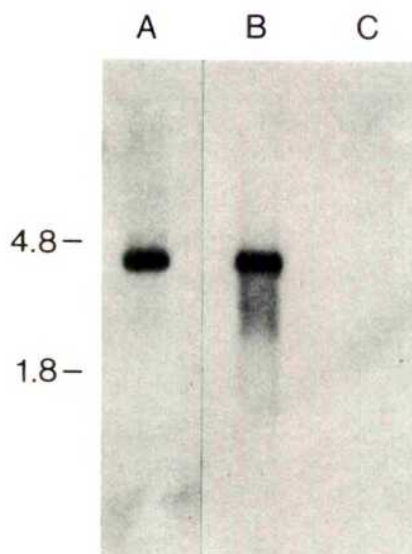


Fig 2. Identification of factor XIII a-chain mRNA by Northern blot analysis. ³²P-radiolabeled cDNA (630-base pair *Eco*RI restriction fragment) was hybridized to poly (A)⁺ mRNA from: lane A, human peripheral blood monocytes (1 μ g); lane B, human placenta (0.01 μ g); lane C, K562 cells (5 μ g).

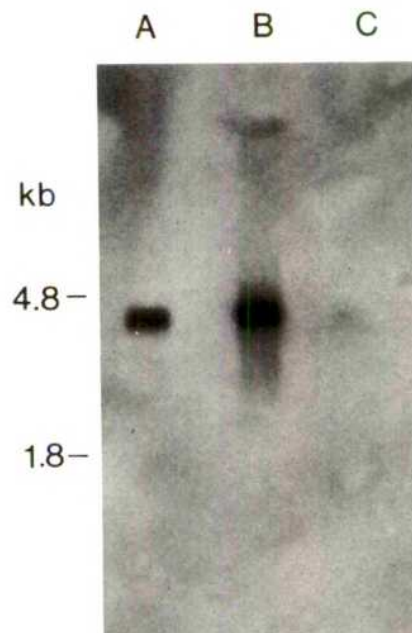


Fig 3. Identification of factor XIII a-chain mRNA by Northern blot analysis. Probe as in Fig 2. Poly (A)⁺ mRNA is from: lane A, human liver (10 μ g); lane B, human placenta (0.09 μ g); lane C, K562 cells (5 μ g).

DISCUSSION

Because monocytes and macrophages are phagocytic cells, identifying factor XIII a-chains intracellularly or on the cell surface would not exclude the possibilities that the a-chains were endocytosed or adsorbed from the plasma rather than synthesized directly. Moreover, a-chains in peripheral blood monocytes may represent protein previously synthesized in bone marrow monocyte precursors rather than in the mature cells themselves. The data of the present study (Fig 2) conclusively demonstrate primary peripheral blood monocytes as a site of synthesis of factor XIII a-chains. The intensity of hybridization of our ³²P-radiolabeled cDNA probe is approximately equal for the monocyte (lane A) and placental (lane B) poly (A)⁺ mRNA samples; since the amount of RNA applied to the gel was 100 times as great from monocytes (1 μ g) as from placenta (0.01 μ g), we estimate that an ~100-fold greater relative abundance of message for factor XIII a-chain exists in placenta than in peripheral blood monocytes. Platelets have factor XIII a-chains,¹ but synthesis of protein by platelets has not been convincingly demonstrated. Even if a small amount of megakaryocyte-derived mRNA persisted in circulating platelets, our monocyte preparation appeared to be entirely free of platelet contamination, thus eliminating this potential cause of false positive results.

To our knowledge, these data represent the first demonstration of synthesis of any blood coagulation factor in primary uncultured and unstimulated monocytes or macrophage cells. Although several studies report identification of factor XIII a-chain protein in such cells,⁵⁻⁷ demonstration of actual synthesis of the protein was performed on a tissue culture cell line (U937, a human monocytelike cell line).^{21,22}

Several studies have reported monocyte-macrophage synthesis of other coagulation factors. Synthesis of factor VII by human alveolar macrophages in vitro was demonstrated on cultured cell monolayers.²³ Increased expression of factor VII protease activity, possibly representing synthesis, was shown in cultured human peripheral blood monocytes stimulated with bacterial lipopolysaccharide, an endotoxin, but not in freshly isolated cells or unstimulated cultured cells.²⁴ Mouse peritoneal macrophages synthesized factors II, VII, IX, and X when grown in tissue culture.²⁵ These previous studies were performed on monocytic cell lines, fresh monocyte or macrophage cells grown in tissue culture, or stimulated cells, to demonstrate synthesis of the clotting factors. Based on our observation that normal, unstimulated monocytes synthesize factor XIII a-chains, this enzyme is likely to have an important function under physiologic conditions.

The function of factor XIII a-chains in circulating monocytes is uncertain. Factor XIII is a transglutaminase enzyme that covalently cross-links fibrin monomers and stabilizes the fibrin clot. In addition, it has several other substrate specificities and has been shown to cross-link collagen,²⁶ fibronectin,²⁷ thrombospondin,²⁸ Factor V,²⁹ and von Willebrand's factor.³⁰ The factor XIII a-chains synthesized in monocytes might be a readily available source of transglutaminase activity in areas of vascular endothelial injury for processes such as clot retraction and wound healing. As monocytes exit the vascular spaces and enter tissues to become macrophages, they may be expected to carry with them the ability to synthesize factor XIII a-chains and to participate in similar processes in areas of tissue inflammation.

Our data demonstrate human liver as a site of synthesis of factor XIII a-chains (Fig 3). More than 100 times as much poly (A)⁺ mRNA from liver (lane A) as from placenta (lane B) was applied to the gel (10 and 0.09 μ g, respectively), yet hybridization was approximately five times more intense for the placental sample. This result indicates that there is an ~500-fold greater relative abundance of message for factor XIII a-chain in the placenta than in the liver. Thus, the results provide a possible explanation for the finding by Grundmann and colleagues,¹³ who reported hybridization of a factor XIII a-chain cDNA fragment to poly (A)⁺ mRNA

from human placenta but not to poly (A)⁺ mRNA from human liver. In that study, equal quantities (4 μ g) of poly (A)⁺ mRNA from each tissue source were probed, and hybridization to the liver RNA appeared to be negative.

Some investigators have found factor XIII a-chain protein in human⁹ and rabbit¹⁰ hepatocytes and the hepatoma cell line Hep G2,⁸ but others^{11,12} did not detect the protein in human hepatocytes. A variety of techniques were used for these studies, including enzyme-labeled antibody staining,⁹ identification of protein by fluorescent antibody binding and measurement of synthesis by radioimmunoassay and [¹⁴C] leucine incorporation in isolated hepatocytes,¹⁰ ¹²⁵I-factor XIII a-chain radioimmunoassay of serum-free culture media,⁸ and immunomorphological and biochemical methods.¹¹ Fear and associates¹² did not find factor XIII a-chains in hepatocytes by an immunoperoxidase technique using rabbit anti-human factor XIII a-chain antibody on sections of paraffin-embedded human liver. They did, however, find a-chains in cells interpreted as inactive fibroblasts in the portal tracts. Our results indicate a very low abundance of message for factor XIII a-chains in liver tissue, most of which is comprised of hepatocytes. If the intracellular concentration of a-chain protein were also very low in hepatocytes, the discrepant findings in the previous studies might be due to different sensitivities of the various techniques used. In addition, since fibroblasts may be very difficult to distinguish from tissue macrophages, it is interesting to speculate that the positively stained cells in the study of Fear and colleagues were actually macrophages. Such cells, if derived from blood monocytes, might have a greater concentration of factor XIII a-chains than do hepatocytes, thus appearing positive by an immunoperoxidase technique while hepatocytes appear negative. To identify the specific cells in liver tissue that synthesize factor XIII a-chain, we have begun work on in situ hybridization using cDNA probes.

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CONCISE REPORT

An ~300 bp Deletion Involving Part of the 5' β -Globin Gene Region Is Observed in Members of a Turkish Family With β -Thalassemia

By J.C. Diaz-Chico, K.G. Yang, A. Kutlar, A.L. Reese, M. Aksoy, and T.H.J. Huisman

Detailed gene mapping analyses of genomic DNA from two Turkish subjects with a β -thalassemia trait demonstrated an ~300 bp deletion, which is located between the *Rsa* I restriction site 128 bp 5' to the Cap site and the *Acc* I restriction site 284 bp 3' to the same Cap site; it includes the 5' β promoter region, the first exon, and (part of) the IVS-I. Heterozygotes for this and two other β -thalassemia

types, which are also caused by deletions involving 5' β promoter sequences, appear to have higher hemoglobin (Hb) A₂ levels, perhaps because the loss of this promoter results in an increased transcription of the δ globin gene, as δ and β promoters may be influenced by the same enhancing sequences 3' to the β globin gene.

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THE β -THALASSEMIAS form a most heterogeneous group of disorders in hemoglobin (Hb) synthesis; nearly 40 are the result of base substitutions, frameshifts, or deletions of a few nucleotides, while only a few are caused by deletions of part or all of the β globin gene (summarized in ref. 1). In this short report we discuss data from detailed gene mapping analysis of a β -thalassemia chromosome present in two members of a Turkish family, in which (part of) the promoter region 5' to the β globin gene and a small segment at the 5' end of this gene were deleted.

MATERIALS AND METHODS

Blood samples. These were collected in vacutainers with EDTA as anticoagulant and transported in ice by air by one of the authors. Informed consent was obtained.

Hematological methods. Hematological data were obtained on freshly collected samples using an automated cell counter. The Hb type was evaluated by cellulose acetate electrophoresis at pH 8.9,² Hb A₂ was quantitated by microcolumn chromatography,³ and Hb F by alkali denaturation.⁴ The G₁ level in isolated Hb F was determined by reversed phase high performance liquid chromatography (HPLC).⁵

DNA methods. DNA was isolated from WBCs by the method of Poncz et al.⁶ Gene mapping followed procedures routinely in use in our laboratory⁷⁻¹⁰; size estimation of different fragments of DNA was facilitated by the use of gels made of 0.8%, 1.0%, 1.2%, 1.6%, 1.8%, 2.0%, and even 2.3% agarose. Haplotyping involved the following restriction sites: *Hinc* II 5' to ϵ ; *Xmn* I 5' to G₁; *Hind* III at G₁ and A₁; *Hinc* II at $\psi\beta$ and 3' to it; *Ava* II at β ; *Hpa* I and *Bam* HI 3' to β .^{8,11-13} Probes used were: ϵ , IVS-II, $\psi\beta$, β IVS-II, and 5' β ; details about these probes have been discussed before,^{10,12,13} while the exact locations of the 5' β and β IVS-II probes are indicated in Fig 1.

RESULTS

The family. The 23-year-old propositus is a Turkish woman, first evaluated in 1978 because of anemia (Hb 10.6 g/dL; PVC 0.36 L/L; MCV 68 fL), thalassemic red cell morphology, and iron deficiency (serum Fe 70 μ g/dL). She was treated with iron; the Hb level after therapy increased to 13.0 g/dL. Her father was diagnosed as having the same condition (Hb 13.0 g/dL; PCV 0.41 L/L; MCV 68 fL), while the mother and younger sister were normal. The family originates from Mersin (southern Turkey); the father might be of Eti-Turk or Arabian origin.

Recent hematological data, listed in Table 1, confirm the earlier observations. Hb F_{AD} were distinctly elevated at about 3% with low G₁ values. Haplotyping was greatly facilitated by the fact that the father was homozygous at all nine

restriction sites (see Materials and Methods); the β Th haplotype was [+ - - - - + + +], which corresponds to haplotype no. I of Orkin et al.¹¹

Gene mapping analysis. A small deletion of ~300 bp was initially detected when a *Bam* HI digest was hybridized with a synthetic oligonucleotide probe of 19 nucleotides, similar to those occupying positions 289 to 307 (inclusive) 3' to the Cap site of the β globin gene [this is the normal control used in identifying the well-known CAG to TAG mutation at codon 39¹⁶⁻¹⁸]. Figure 2 shows the presence of a 1.6 kilobase (kb) fragment in addition to the normal 1.9 kb fragment. Comparable smaller fragments were seen in *Bam* HI and *Ava* II digests (5' β probe) and in *Bgl* II, *Pst* I, *Eco* RI, *Hinc* II, and *Sph* I digests (β IVS-II probe), while a larger fragment of ~8.3 kb was observed in an *Nco* I digest (Fig 2). These initial results located the 0.3 kb deletion between the *Sph* I site (614 bp 5' to the Cap site) and the *Ava* II site at the 5' end of the exon 2 of the β globin gene (Fig 1). This location was confirmed by data from the *Hgi* AI digest (5' β probe). Digests from control DNA samples contained, besides the expected larger 4.7 kb and 4.1 kb fragments, smaller fragments of 1.05, 0.72, and 0.33 kb; their presence was determined by the presence or absence of the polymorphic site about 0.06 kb 3' to the Cap site.¹¹ Each of the two patients had a slightly different fragment of 0.75 kb suggesting that the polymorphic *Hgi* AI site is part of the deletion. The

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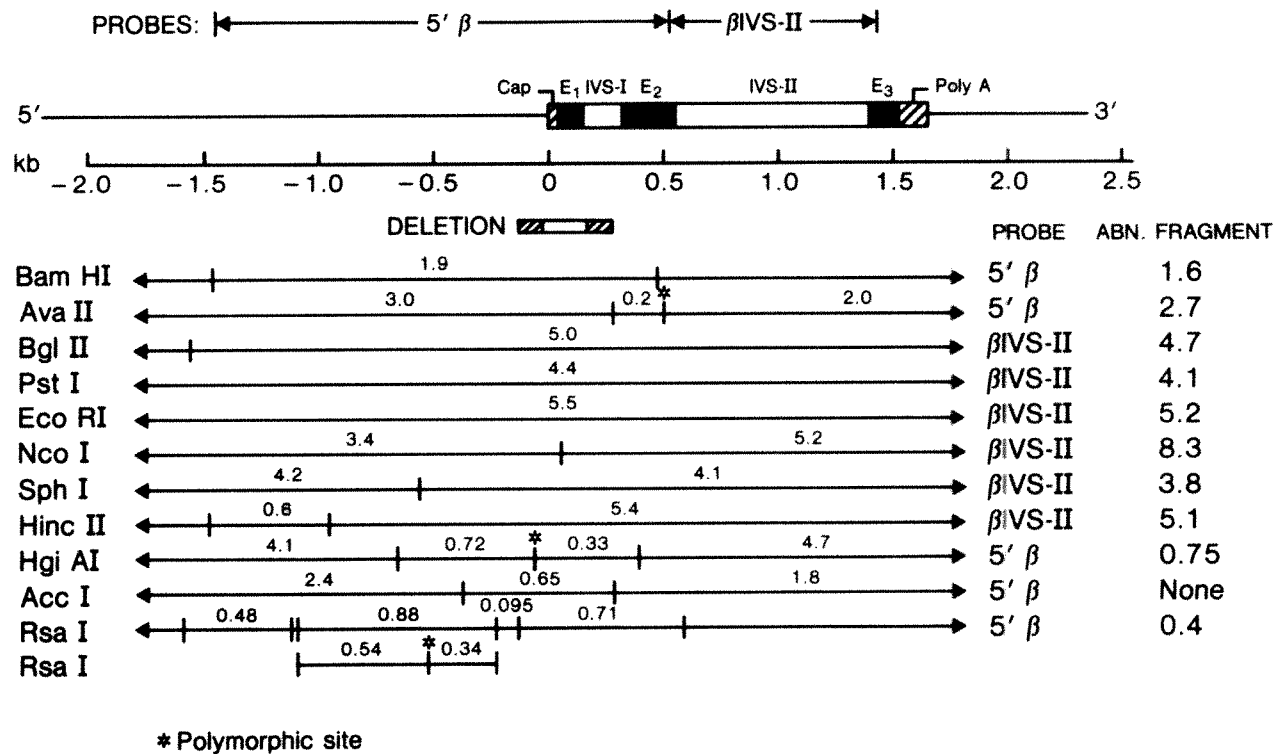


Fig 1. Map of the β globin gene with sites of 11 restriction endonucleases. The restriction sites were obtained from the sequence data of the β globin gene region.^{14,15}

normal chromosome of the propositus A33 did not have the polymorphic site as only a 1.05 kb fragment was observed, while the 0.72 and 0.33 kb fragments in the DNA of the father A39 indicated that this site was present on his normal chromosome.

Additional data were obtained from the *Acc I* and *Rsa I* digests. No abnormal fragment was present in the *Acc I* digests, but the intensity of the 0.65 kb fragment was about one half that of the control (Fig 3) suggesting that the ~0.3 kb deletion occurred between the two *Acc I* sites, 0.37 kb 5' to the Cap site and 0.28 kb 3' to the Cap site (Fig 1). Several small fragments were present in an *Rsa I* digest that could be separated on gels with different agarose concentrations; best results were obtained on 1.6% and 2.0% agarose gels (Fig 3). The data are somewhat complicated by the presence of a polymorphic site ~0.5 kb 5' to the Cap site, which splits the expected 0.88 kb fragment into a 0.54 kb fragment and a 0.34 kb fragment (Fig 1).¹⁹⁻²¹ Thus, fragments observed in DNA without this restriction site on both chromosomes are: 0.88, 0.71, and 0.48 (the small 0.095 kb fragment is not

detected), while the fragments in DNA with this restriction site on both chromosomes are: 0.71, 0.54, 0.48, and 0.34 (in Fig 3: Control C₁ is -/-; C₂ is +/-). Subjects A33 and A39 both showed an additional fragment of 0.41 kb, while A39 was found -/- and A33 +/- for the polymorphic *Rsa I* site. These data indicate that the ~0.3 kb deletion is located between the *Rsa I* sites 128 bp 5' and 581 bp 3' to the Cap site. These results, together with those of the *Acc I* digest, locate the ~0.3 kb deletion between the *Rsa I* site 128 bp 5' and the *Acc I* site 284 bp 3' to the Cap site (Fig 1).

DISCUSSION

The location of the ~300 bp deletion in this Turkish β thalassemia between the *Rsa I* site, which is 128 bp 5' to the Cap site and the *Acc I* site, and which is found 284 bp 3' to the Cap site and within the 5' end of exon 2 (see Fig 1), indicates that the deletion includes exon 1, (part of) the IVS-I, and promoter sequences, 5' to the β globin gene. A β^0 -type of thalassemia will be the result with hematological abnormalities, which in the heterozygote, include mild ane-

Table 1. Hematological and Hemoglobin Composition Data

Case	Sex/Age	Relation	D _x	Hb g/dL	PCV L/L	RBC 10 ¹² /L	MCV fL	MCH pg	MCHC g/dL	A ₂ * (%)	F _{AD} † (%)	G ₂ ‡ (%)
A39	M/58	Father	A β Th	14.4	.435	6.52	67	22.1	33.1	8.1	2.7	20.4
A41	F/45	Mother	AA	13.2	.430	4.62	93	28.6	30.7	2.8	0.5	—
A33	F/23	Propositus	A β Th	11.8	.360	3.33	68	22.1	32.8	7.1	3.3	13.1
A40	F/21	Sister	AA	12.6	.370	4.10	90	30.7	34.1	2.8	0.7	—

*By microcolumn chromatography.³

†By alkali denaturation.⁴

‡By reversed phase HPLC.⁵

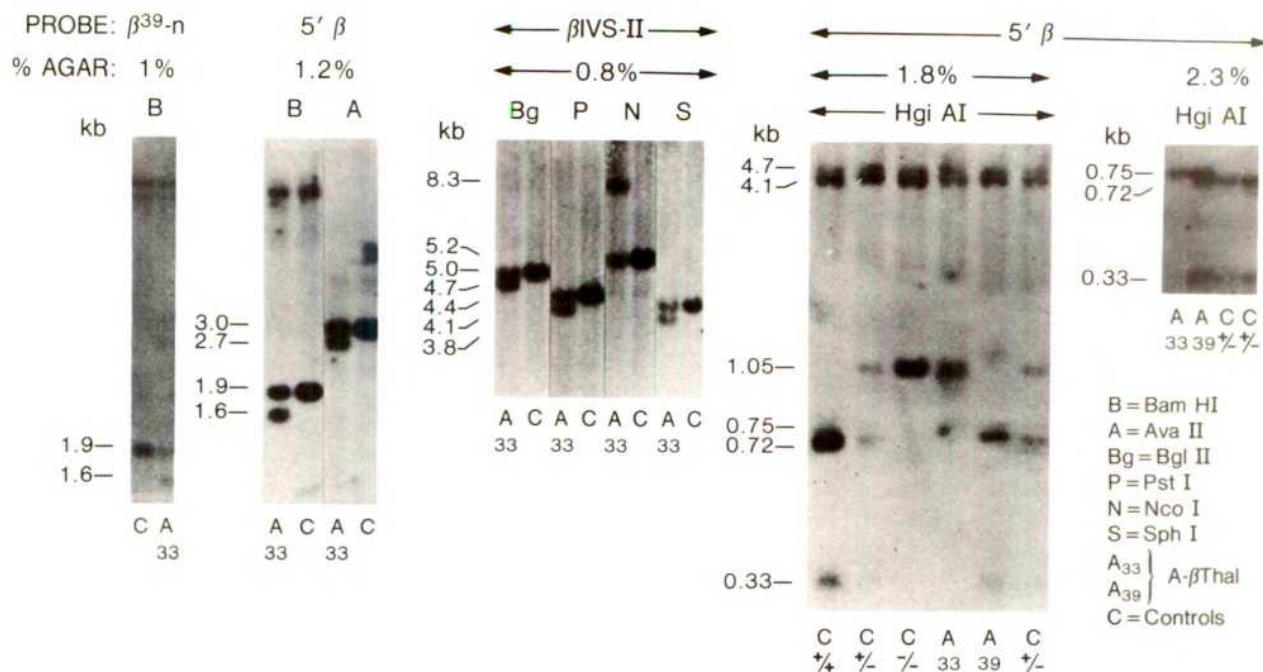


Fig 2. Identification of abnormal β -globin gene fragments in β thalassemia DNA. The patients A33 and A39 are listed in Table 1.

mia, rather severe microcytosis, and elevated Hb A₂ and Hb F values (Table 1). This newly discovered deletion is the fifth reported thus far; the others are the Asian Indian type with a 619 bp deletion including the 3' end of the IVS-II and the entire exon 3,²²⁻²⁵ the American black type with an ~1,350 bp deletion involving ~600 bp 5' to β , the exon 1, IVS-I, exon 2, and part of IVS-II,⁹ the Czechoslovakian type with a 4,237 bp deletion involving nearly the entire β globin gene,²⁶ and the Dutch type with an ~12,500 bp deletion, which includes the entire β globin gene.^{10,19} Clinical and hematological abnormalities in persons heterozygous for either one of the five deletions are almost identical except for a rather high Hb F level in the Dutch heterozygotes.

In their article defining the Czechoslovakian β^0 -thalasse-

mia, Popovich et al²⁶ emphasize the high Hb A₂ levels of 8.9 to 9.0 observed in these heterozygotes (using a microcolumn chromatographic method), and a comparison of similar data for the other β^0 -thalassemia heterozygotes with deletions of the 5' region of the β globin gene made them suggest that such a deletion is associated with a higher δ chain synthesis. The data for our two β^0 -thalassemia heterozygotes appear to support this conclusion; the levels in our patients were 7.1% and 8.1%, which correspond to absolute values of 1.57 and 1.79 pg/cell (average value for normal controls: 0.75 pg/cell). Also, the Hb A₂ value in the American black with a heterozygosity for the ~1,350 bp deletion was recently reevaluated in freshly collected samples and was found to be 7.3% and 7.0%, similar to the 7.1% reported earlier (or 1.60

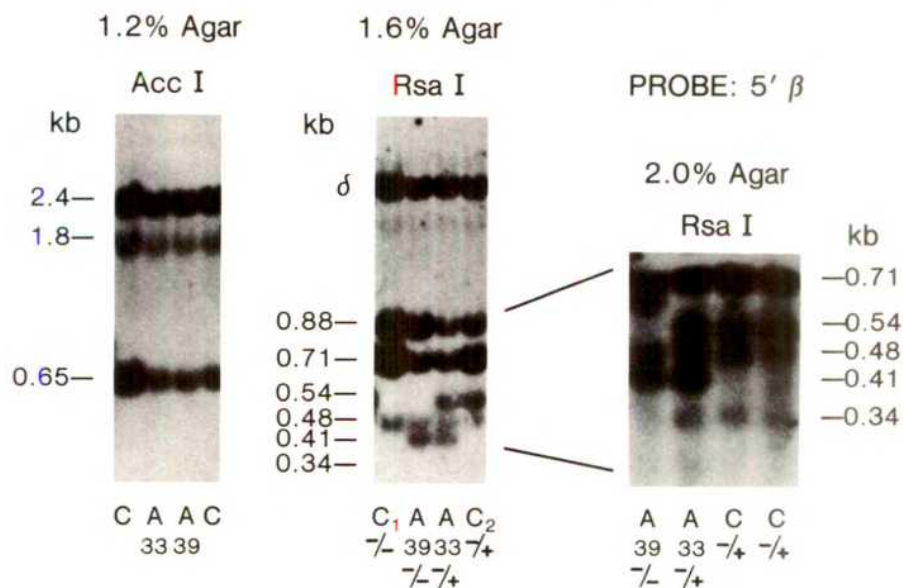


Fig 3. Identification of *Acc* I and *Rsa* I fragments using the 5' β probe. A33 and A39 are the two patients listed in Table 1.

pg/cell).⁹ In contrast, average Hb A₂ values by microcolumn chromatography for 40 persons with a β^0 thalassemia due to a CAG to TAG nonsense mutation at codon β 39¹⁶ averaged 4.75% with a range of 3.5% to 6.8% corresponding to 1.13 pg/cell (with a range of 0.84 to 1.43 pg; unpublished data). Thus, our limited additional data lend support to the suggestion by Popovich et al²⁶ and appear to indicate that the loss of the 5' β promoter indirectly affects the transcription of the δ globin gene, assuming that both the 5' δ and 5' β promoters are influenced by the same enhancer, located 3' to the β globin gene.^{28,29} If this hypothesis is correct, one would expect relatively low Hb A₂ values in Indian β -thalassemia heterozygotes because the deletion of 619 bp includes DNA some 150 bp 3' to the poly-A site. However, it is unknown if the enhancer sequence is left intact as it may be located 3' to this

deletion. We recently studied two such Indian families, living in the Canary Islands; the Hb A₂ values in two heterozygotes were 6.5% and 6.8% (unpublished data).

Another interesting feature in these three types of deletional β^0 -thalassemia is the relatively high Hb F_{AD} level of 3.3%, 5.0%, and 5.7% in the Czechoslovakian heterozygotes, of 7.1% in the black heterozygote, and of 2.7% and 3.3% in the two Turkish heterozygotes. The several heterozygotes for the Dutch type of β^0 -thalassemia had Hb F_{AD} values varying between 4.1% and 10.9%.¹⁰ It may well be that the unusual increase in δ chain production is accompanied by a somewhat similar increase in γ chain (both G _{γ} and A _{γ}) production; the Hb F_{AD} value in the 40 β -thalassemia heterozygotes with the β 39 nonsense mutation averaged only 1.2% with a range of 0.1% to 5.7% (unpublished data).

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CONCISE REPORT

Bone Marrow Transplantation for Patients With Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

By S.J. Forman, M.R. O'Donnell, A.P. Nademanee, D.S. Snyder, P.J. Bierman, G.M. Schmidt, J.L. Fahey, A.S. Stein, P.M. Parker, and K.G. Blume

We report the treatment outcome of allogeneic bone marrow transplantation in ten patients with Philadelphia chromosome-positive acute lymphoblastic leukemia. Six patients are alive and well for 6 to 30 months (median 19 months) after transplantation. Four patients died with transplant related complications. In view of the poor prog-

nosis associated with this disease, marrow ablation followed by allogeneic or syngeneic marrow grafting may be the preferred treatment modality if a suitable marrow donor is available.

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PHILADELPHIA CHROMOSOME (Phl)-positive acute lymphoblastic leukemia (ALL) is a variant of ALL that is associated with an exceptionally poor prognosis.^{1,2} Although complete remissions (CR) are readily attained with remission induction chemotherapy in ~70% of adult patients with this disorder, median remission duration is <1 year. In addition, long-term, disease-free survival is rare even when intensification and maintenance regimens that have been successful in other variants of ALL are administered. To overcome the resistant nature of the abnormal clone involved, bone marrow ablation with high-dose radiochemotherapy followed by bone marrow transplantation (BMT) has been used in some patients.^{3,4} We report our experience using allogeneic BMT as a treatment for ten adult patients with Phl-positive ALL.

MATERIALS AND METHODS

Preparation for BMT consisted of fractionated total body irradiation (FTBI) with 1320 cGy (11 × 120 cGy on day -7 through day -4 at a dose rate of 15 cGy/min)⁵ in eight patients and of single-dose total body irradiation (STBI) with 750 cGy at 26 cGy/min on day -5 in one patient.⁶ This was followed on day -3 by intravenous (IV) administration of cyclophosphamide (CY) at a dose of 100 mg/kg⁷ in three patients or etoposide (VP16) at a dose of 60 mg/kg⁸ in six patients. For technical reasons, one patient was treated with busulfan (BU) at 16 mg/kg given over 4 days (day -7 through day -4) followed by CY at 60 mg/kg administered on day -3 and day -2.⁹ Supportive care was provided as previously described.¹⁰ The BMT procedure from the histocompatible sibling donors was carried out on day 0 following a generally practiced method.¹¹ To prevent graft-v-host disease (GVHD), patients received either cyclosporine A/prednisone (CSA/PSE) or methotrexate/prednisone (MTX/PSE); details of the two drug schedules, investigated in a prospective randomized GVHD study, were reported recently.¹²

Patients. Between October 1984 and October 1986, 10 patients with Phl-positive ALL were accepted for BMT at the City of Hope National Medical Center. At the time of entry, patients were assigned a unique patient number (UPN). Initially the WBC cell count of these patients ranged from 400 to 350,000/μL (median: 7,975/μL). The distribution of immunophenotypes of ALL was as follows: null type (1 patient), common ALL antigen (CALLA) positive (3 patients) and pre-B type (6 patients). Because these patients had been referred to us from eight different leukemia centers, their initial chemotherapy had contained various drug combinations. The patients had been treated with the following chemotherapeutic agents: daunorubicin (9 patients), vincristine (9 patients), PSE (8 patients), L-asparaginase (6 patients), cytosine arabinoside (5 patients), MTX (5 patients), 6-mercaptopurine (4

patients), 6-thioguanine (2 patients), and CY (2 patients). Eight patients had attained CR within 4 to 8 weeks after beginning remission induction therapy, whereas 2 others had failed repeated remission induction attempts. When the preparatory regimen was administered, 4 patients were in first CR, 2 had failed to enter CR, 1 was in first relapse, and 3 were refractory to reinduction attempts. All clinical research protocols have been approved by the Institutional Review Board of the City of Hope Medical Center. Patient entry to the study was contingent upon prior written informed consent.

RESULTS

By the end of the first month following BMT, all patients showed signs of engraftment, documented by appropriate genetic marker analysis.¹³ One patient (UPN 323) has become a mixed lymphohematopoietic chimera.¹⁴ Five patients showed no signs of acute GVHD, whereas in two patients GVHD was mild (grade I) and in three patients it was moderate to severe (grades II through IV).

Six patients are currently alive and in continued CR for 6 to 30 months (median 19 months) after BMT. The performance status¹⁵ of the six surviving patients is 80 to 100 (median 100); one of them (UPN 271) has mild chronic GVHD. Four patients died within the first 4 months following marrow grafting: 2 with GVHD and interstitial pneumonia (IP), 1 with polymicrobial infection, and 1 with a combination of veno-occlusive disease (VOD), bleeding, and fungemia. Details of the ten BMT recipients on this study are shown in Table 1.

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Table 1. Bone Marrow Transplantation for Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

UPN	Age/Sex	Initial WBC/ μ L	Immuno-phenotype	DX $\rightarrow$ CR (wk)	CR $\rightarrow$ BMT (mo)	Status at BMT	Blasts in BM (%)	Preparatory Regimen	GVHD Prophylaxis	Acute GVHD	Outcome
264	23/M	8,900	CALLA	4	3	1.CR	< 5	FTBI/CY	CsA/PSE	0	Alive in CCR, day + 892
271	28/M	1,500	Null	4	5	1.CR	< 5	FTBI/CY	CsA/PSE	I	Alive in CCR, day + 836
323	28/M	7,400	PRE-B	4	2	1.CR	< 5	FTBI/CY	CsA/PSE	I	Alive in CCR, day + 523
397	29/F	2,900	PRE-B	4	2	1.CR	< 5	FTBI/VP 16	CsA/PSE	0	Died with polymicrobial infection on day + 63
304	36/M	4,600	CALLA	NA	NA	IF	8	FTBI/VP 16	MTX/PSE	II	Died with GVHD/IP and sepsis on day + 121
306	25/M	350,000	PRE-B	4	4	1.RIF	14	FTBI/VP 16	MTX/PSE	III	Alive in CCR on day + 620
309	23/M	315,000	PRE-B	NA	NA	IF	20	STBI/VP 16	CsA/PSE	IV	Died with GVHD/IP on day + 93
353	42/M	400	CALLA	8	14	1.RIF	40	BU/CY	CsA/PSE	0	Died with VOD, GI bleeding, and fungemia on day + 36
355	23/M	8,550	PRE-B	4	3	1.REL	23	FTBI/VP 16	CsA/PSE	0	Alive in CCR on day + 335
392	45/M	20,000	PRE-B	6	22	1.RIF	10	FTBI/VP 16	CsA/PSE	0	Alive in CCR on day + 171

Date of evaluation: April 3, 1987.

Abbreviations: UPN, unique patient number; DX, diagnosis; NA, not applicable (because patient failed to attain CR); IF, induction failure; RIF, reinduction failure; REL, relapse; STBI, single-dose total body irradiation; VP 16, etoposide; CCR, continued CR.

DISCUSSION

Six of 10 patients with Phl-positive ALL were transplanted successfully with marrow from histocompatible siblings, whereas four patients died with BMT-related complications. Three of the four patients who had undergone BMT during first CR are surviving; the other three survivors were in more advanced stages of their disease when preparation for BMT was begun.

There are several caveats regarding our study: The patient

group under study is relatively small and the observation time is still limited to 6 to 30 months. Neither can we conclude from our data the preparatory regimen to recommend. This issue must be addressed in future studies.

Nevertheless, our data, which require confirmation by other transplant centers, can be viewed as encouraging. Indeed, considering the poor prognosis for patients with this variant of ALL, BMT performed early during the course of this disease may currently be the treatment of choice if a suitable donor is available.

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CONCISE REPORT

Use of Laser-UV for Inactivation of Virus in Blood Products

By Kristina N. Prodou, Joseph C. Fratantoni, Elizabeth J. Boone, and Robert F. Bonner

Inactivation of virus by UV radiation was examined as a potential method for sterilization of blood products. Samples of attenuated poliovirus, platelets and plasma were uniformly irradiated with a XeCl excimer laser that delivered 40 nsec pulses of UV at 308 nm (UVB₃₀₈). Intensities and exposure doses were varied from 0.11 to 1.40 MW/cm² and 0.51 to 56.0 J/cm², respectively.* In studies conducted with low intensity UVB₃₀₈ (≤ 0.17 MW/cm²), using exposure doses ≥ 10.8 J/cm², it was possible to inactivate poliovirus by 4 to 6 log₁₀. Platelets irradiated with doses ≤ 21.5 J/cm² exhibited minimal damage as assessed by aggregation activity and spontaneous release of serotonin. Examination of the coagulation activity of irradiated plasma indicated that exposure doses ≤ 21.5 J/cm² resulted in

$<20\%$ increase in prothrombin and partial thromboplastin times. The use of UVB₃₀₈ at a higher intensity (1.4 MW/cm²) over a similar range of exposure doses did not enhance viral inactivation but did result in increased damage to platelet and plasma proteins. These results demonstrate that at 308 nm there exists a "window of efficacy" for exposure doses between 10.8 and 21.5 J/cm² and peak intensities ≤ 0.17 MW/cm² in which a hardy virus is significantly inactivated and platelets and plasma proteins are, by functional criteria, minimally affected. Increased viral inactivation cannot be accomplished with higher UV intensities and will require additional or alternate measures.

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AS PART OF the effort to reduce transmission of viral disease by blood products, donors are routinely screened for hepatitis B surface antigen (HB_sAg) and for antibody to human immunodeficiency virus (HIV). Although such screening has been successful in providing a safer blood supply, the potential extent of this approach is limited. Screening procedures are expensive, time-consuming and, since some are necessarily hypersensitive, they result in loss of false positive donors. Finally, not all donors infected with HB_sAg or HIV are detected and there are no direct tests to screen for non-A, non-B hepatitis or for other known and emerging agents potentially transmissible by blood.

The early studies of Murray et al showed that doses of UV radiation that were sufficient to inactivate hepatitis virus caused unacceptable plasma protein denaturation.¹ Subsequently, a variety of physical and chemical agents, alone and in combination, have been demonstrated to inactivate virus while preserving some degree of plasma protein function, but no similar demonstration has been offered for cellular blood components.²⁻⁴

Since the cells most often transfused are anucleate and nonreplicating, appropriate conditions of UV irradiation should favor photo-induced damage of viral nucleic acid with minimal effects on cellular proteins. In this report, we describe results of the treatment of attenuated poliovirus (chosen as a model of a hardy, RNA virus), platelets and plasma with pulsed UV radiation at 308 nm. The demonstra-

tion of a dose-dependent decrease in virus titers with radiation doses that only moderately affected platelet and plasma function indicates that UV irradiation is a potential method for reduction of the viral bioburden of transfusable blood products.

MATERIALS AND METHODS

A XeCl excimer laser (Hyperex 460, Lumonics Inc, Kanata [Ottawa], Ontario, Canada) was used as the source of radiation at a fixed wavelength of 308 nm (the UVB region of the spectrum). Pulses (40 nsec) were delivered at rates of one to 83 pulses per second with low (0.11 to 0.17 MW/cm²) and high (1.4 MW/cm²) intensities. Exposure doses (10 to 1,000 pulses) were varied from 0.51 to 56.0 J/cm². One-milliliter samples of material to be irradiated were placed in 5-mL polyethylene tubes (Falcon no. 2063, Sarstedt, Inc, Princeton, NJ) and rotated during exposure in the beam at a fixed distance from the source. The rotation of the sample partially smooths out the nonuniformity of the sample irradiance due to absorption within the plasma or buffer with albumin. The irradiance within the sample decreased 3.8-fold from the surface of the sample to the center (see Discussion).

Platelets obtained from whole blood were prepared as previously described⁵ with the following modifications: platelet wash buffer, pH 7.40, contained 137 mmol/L NaCl, 2.7 mmol/L KCl, 0.5 mmol/L NaH₂PO₄, 12 mmol/L NaH₂CO₃, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, and 50 g/L human albumin (ICN Immunobiologicals, Lisle, IL). Prostaglandin E₁ (Sigma Chemical Co, St Louis), final concentration 1 μ mol/L was added to the platelet rich plasma and the first wash. The washed platelets were resuspended in wash buffer containing 0.5 mmol/L CaCl₂. One-milliliter samples of the platelet

*Glossary of terms:

Photon: a quantum of light energy that is transferred to an absorbing molecule. For 308 nm, the energy of photons is equal to 92.8 kcal/mol.

Intensity (or sample irradiance): the power (or number of photons per second) of light per unit (incident on a sample). Expressed as megawatts (MW) per square centimeter; (eg, 1 MW/cm² = 6.2 photons at 308 nm incident on each square angstrom within 40 nanoseconds [nsec]).

Exposure dose (or energy density): the cumulative energy incident on a sample per unit area; the product of intensity and total exposure duration. Expressed as joules (J) per square centimeter (eg, 1 J = 1 watt for 1 sec). In this study, 0.5 to 50 J/cm² is equivalent to 78 to 7,800 photons at 308 nm per square angstrom.

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suspension ($400,000/\mu\text{L}$) were subjected to UVB_{308} as described above.

Sabin's attenuated poliovirus type 1 was suspended in platelet wash buffer containing 50 g/L human serum albumin and 1-mL samples subjected to UVB_{308} as described above. Poliovirus activity was determined by virus titration on Vero cell cultures grown in microtiter trays.⁶ Titers are expressed as infectious doses (50%) in 0.05 mL of experimental material.

Spontaneously released serotonin was assayed as previously described.⁵ Before platelet washing, platelet rich plasma (PRP) was incubated with $0.05 \mu\text{Ci/mL}$ ^{14}C -serotonin (New England Nuclear, Boston, MA) for 30 minutes at 37°C . Imipramine ($3.4 \mu\text{mol/L}$) was added to the final platelet suspension to prevent re-uptake of serotonin following irradiation. Results are expressed as percent of releasable serotonin remaining in the platelets. The value for 100% releasable serotonin was determined by stimulating an aliquot of labeled platelets with $5 \mu\text{mol/L}$ A23187 calcium ionophore.

The aggregation response of washed platelets following UVB was measured on a dual channel aggregometer (Payton Associates, Inc, Buffalo). Platelet suspensions were incubated at 37°C for 30 minutes post irradiation, then stirred at 1,000 rpm for 30 seconds before the addition of $164 \mu\text{g/mL}$ calf skin collagen (Cooper Biomedical Inc, Malvern, PA). The optical density of the nonirradiated platelet suspension was used to set the baseline for all aggregation response experiments. This allowed monitoring of "baseline shift," which reflected the formation of microaggregates in some irradiated samples before addition of collagen.

Plasma was prepared from citrated blood by centrifugation at $4,800 \times g$ for ten minutes. One-milliliter samples were subjected to UVB_{308} , as described above, then held on ice. Prothrombin times and partial thromboplastin times were determined on 0.1-mL samples using standard procedures. Prothrombin time reagents were obtained from Ortho Diagnostic Systems, Inc, Raritan, NJ. Control values ranged from 12.8 to 14.6 seconds. Activated partial thromboplastin time reagents were obtained from Organon Teknika Corp, Morris Plains, NJ. Control values ranged from 29.9 to 40.7 seconds.

RESULTS

The intensity and dose dependence of 308 nm irradiation on washed platelets is illustrated in Fig 1A. At the lower intensities (0.11 to 0.17 MW/cm^2), the spontaneous release of ^{14}C -serotonin was minimal over the range of exposure doses applied (0.51 to 53.7 J/cm^2). Platelets treated with a UVB_{308} dose of 2.15 J/cm^2 retained approximately 80% of the releasable serotonin compared with 85% in the nonirradiated control. However, increasing the laser intensity to 1.4 mW/cm^2 and delivering a similar range of exposure doses resulted in an increased dose-dependent release of serotonin. Platelets that received a UVB_{308} dose of 22.4 J/cm^2 at the higher intensity retained only 50.5% of the releasable serotonin.

Attenuated poliovirus in buffered medium (+5% albumin) was inactivated in a dose-dependent manner by 308 nm irradiation at doses ranging from 0.51 to 56 J/cm^2 (Fig 1B). In contrast to the UVB_{308} effect on platelets, inactivation of poliovirus was not dependent on laser intensity. Virus activity was decreased by 4 to 6 $\log_{10}$ with low intensity (0.11 to 0.17 MW/cm^2) 308 nm irradiation at doses of 10.8 to 22.4 J/cm^2 . Results were similar with high intensity UVB_{308} .

Platelets irradiated with the lower intensity aggregated in response to $164 \mu\text{g/mL}$ collagen (Fig 2) over an exposure dose range of 2.1 to 21.5 J/cm^2 . At the highest UVB_{308} dose,

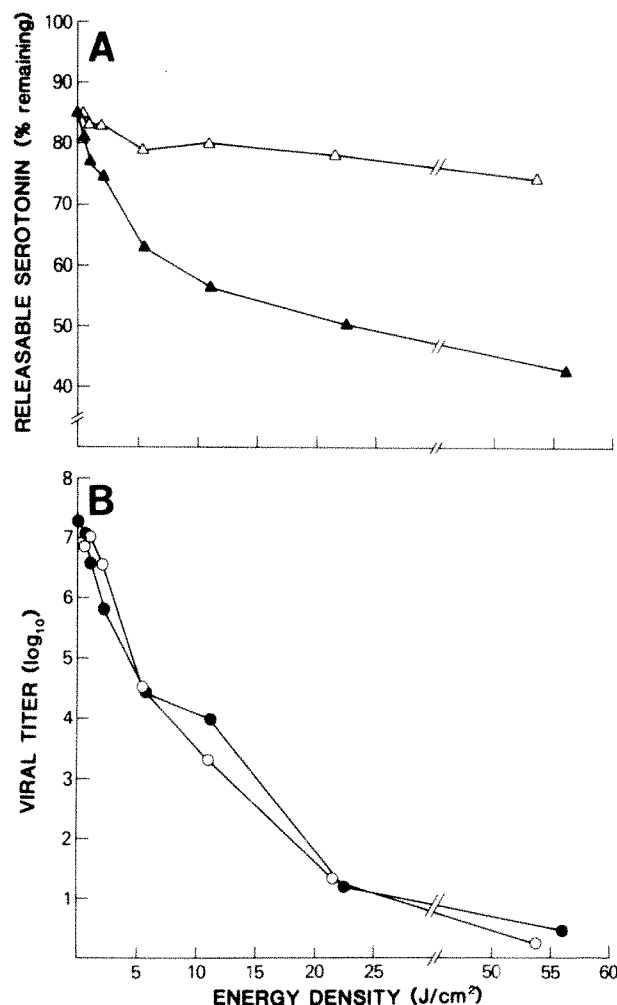


Fig 1. Effect of identical doses of UVB_{308} on poliovirus and platelets. UVB_{308} doses ranged from 0.51 to 53.7 J/cm^2 at low intensities (0.11 to 0.17 MW/cm^2) and 0.56 to 56.0 at high intensity (1.4 MW/cm^2). (A) Spontaneous release of serotonin is illustrated as the percent releasable ^{14}C -serotonin remaining in platelets treated with low (Δ) and high ($\blacktriangle$) intensity UVB_{308} . Nonirradiated control platelets retained 85% of releasable serotonin. (B) Poliovirus titers are expressed as 50% tissue culture infectious doses per 0.05 mL following irradiation with low ($\circ$) and high ($\bullet$) intensity UVB_{308} . Control titer = $7.28 \log_{10}/0.05 \text{ mL}$.

the amplitude of aggregation was decreased to 50% of the control amplitude. A marked loss of aggregation activity was observed at lower UVB_{308} doses with higher peak intensity (1.4 MW/cm^2).

A shift in baseline optical density was noted in platelets treated with energy densities $\geq 21.5 \text{ J/cm}^2$ at lower intensities and in samples treated with $\geq 5 \text{ J/cm}^2$ at the higher intensity. This reproducible shift reflected the presence of microaggregates, as confirmed by phase microscopy. The addition of $50 \mu\text{mol/L}$ prostaglandin I_2 or 2 mmol/L EDTA to the platelet suspension before irradiation did not prevent the formation of microaggregates. However, warming irradiated platelet samples to 37°C for one hour partially reversed the shift in baseline optical density (data not shown).

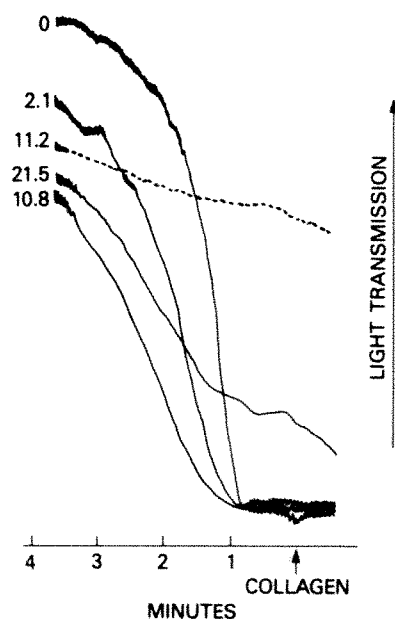


Fig 2. Effect of UVB₃₀₈ on the platelet aggregation response to collagen (164 µg/mL). Values to the left of each curve indicate the UVB₃₀₈ dose (J/cm²) applied. Solid curves represent aggregation activity following low intensity (0.11 to 0.17 MW/cm²) UVB₃₀₈. The dashed curve illustrates the platelet response to collagen following treatment with high intensity (1.4 MW/cm²) UVB₃₀₈ at a dose of 11.2 J/cm².

The plasma prothrombin (PT) and activated partial thromboplastin (PTT) times were assayed as general indicators of radiation effects on plasma proteins. With low intensity UVB₃₀₈, both tests showed dose-dependent increases on irradiation with energy densities ≥ 5 J/cm². Plasma samples irradiated with approximately 10 J/cm² ($n = 3$) demonstrated an average increase of 9.6% over control values for PT and 7.4% for PTT. With a UVB₃₀₈ dose of 20 J/cm² ($n = 3$), the average increases were 19% and 17% for PT and PTT, respectively. At the same dose and a higher peak intensity (1.4 MW/cm²), PT and PTT were elevated 27% and 40%, respectively, above control values.

DISCUSSION

The results of this study demonstrate the inactivation of a hardy virus by pulsed UVB₃₀₈ with rigid control of radiation parameters and mode of delivery. In separate experiments with low intensity UVB₃₀₈, at exposure doses of 10.8 and 21.5 J/cm², poliovirus titers were decreased by 4 to 6 log₁₀, while in vitro assays of platelets and plasma indicate persistence of adequate levels of function. Although the clinical hemostatic efficacy of irradiated platelets was not determined, the degree of modification of the in vitro functions measured is not unlike that seen in platelets stored for periods up to five days after collection.⁷ In our experiments, low intensity UVB₃₀₈ at energy densities ≥ 21.5 J/cm² caused the spontaneous release of only 5% to 10% of releasable serotonin from platelets. Although a diminished aggregation response to collagen with UVB₃₀₈ doses of 10.8 to 21.5 J/cm² was observed, the platelets responded to a single agonist, while stored platelets often exhibit only synergistic aggregation responses.⁷

The excimer laser used in this study permitted very high intensities (up to 2 MW/cm²) at its single wavelength of 308 nm. If UV irradiation of stored blood products is to be practical, it must employ a wavelength that is not highly attenuated by the plastics used in the storage containers or by plasma proteins over a path length of approximately 1 cm. For wavelengths below 300 nm, these attenuations are very large, but diminish rapidly with increasing wavelength above 300 nm. On the other hand, nucleic acid photochemistry is dependent on absorption of the irradiation by the nitrogenous bases that also diminish rapidly with increasing wavelengths above 300 nm. Thus, the 308 nm of the XeCl excimer laser is near the optimum for treatment of plasma and platelets within their sterile storage containers. Based on our results, a sample with thickness of 1 cm (either a planar sheet, as in a blood bag, or a tube) could be treated effectively by uniform irradiation over its entire surface with 10 to 20 J/cm² at 308 nm at peak intensities below 0.2 MW/cm². At 80 pulses per second, our laser could deliver these doses in as little as 25 seconds. The uniformity of sample irradiance could be increased either by mixing the sample during exposure or by reducing the sample thickness. The latter would reduce the volume treated at a given time.

The very high intensities of the excimer laser allow the possibility of exciting a nucleic acid molecule with two photons within the lifetime of an excited state, leading to new, more energetic photochemical products⁸ in addition to pyrimidine dimers produced by single photon absorption. These multiple photon processes in thymine molecules have been reported at intensities >0.8 MW/cm² using 10 nsec pulses at 266 nm.⁸ Such additional nucleic acid damage might be associated with significantly increased virucidal action.

At diminished excimer intensities (0.1 to 0.2 MW/cm²), our results are consistent with virucidal effects reported for much lower intensity UVB radiation,³ which causes single photon photochemical effects in nucleic acids.⁹ We saw no increase in virucidal efficacy by increasing the laser intensity approximately tenfold to 1.4 MW/cm² while maintaining the same cumulative dose (0.51 to 56 J/cm²). However, this same increase in intensity significantly increased damage to coagulation proteins and platelets, presumably by multiple photon excitation of tryptophan residues.¹⁰ Marked alteration of some platelet proteins at these higher intensities (dose ≥ 20 J/cm²) was observed as reduction of specific Ag⁺-staining bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). Thus, at 308 nm we observed selective virucidal effects in plasma and platelets at intensities ≤ 0.17 MW/cm², but at higher intensities this selectivity is diminished by a multiple photon mechanism of protein damage. Procedures that further selectively enhance nucleic acid damage may require use of photoactivating agents as well as modification of radiation wavelength and pulse rate.

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CONCISE REPORT

The 3' Ends of the Deletions of Spanish $\delta\beta^0$ -Thalassemia and Black HPFH 1 and 2 Lie Within 17 Kilobases

By C. Camaschella, A. Serra, G. Saglio, M. Baiget, N. Malgaretti, R. Mantovani, and S. Ottolenghi

Spanish $\delta\beta^0$ -thalassemia, a mild thalassemic condition characterized by increased level of hemoglobin (Hb) F production during adult life, is known to be due to a large deletion starting within the β globin gene cluster and extending beyond the 3' breakpoint of any other similar deletional defects so far identified. By molecular cloning and by genomic mapping we now demonstrate that the deletion of Spanish $\delta\beta^0$ -thalassemia ends at approximately 11 and 17 kilobases (kb) downstream to the 3' endpoints of

black hereditary persistence of fetal hemoglobin (HPFH) type 1 and 2, respectively. As suggested by the complete characterization of this and other deletional defects involving the β globin gene cluster, the 5' and 3' breakpoints of several deletions cluster in rather restricted DNA areas, further strengthening the idea that common molecular mechanisms may operate in causing these deletions.

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KNOWN DELETIONS in the β globin gene cluster span a total DNA length of up to 200 kilobases (kb). These deletions cause a variety of hematological phenotypes including $\gamma\delta\beta$ -thalassemia, $\delta\beta$ -thalassemia, and hereditary persistence of fetal hemoglobin (HPFH)¹⁻²⁵; the latter two conditions are characterized by increased levels of fetal hemoglobin (HbF) during adult age ranging between 5% and 15% in $\delta\beta$ -thalassemia and 20% and 30% in HPFH in heterozygous state.²⁶

The study of the location of the endpoints of several of these deletions has been useful for delineating possible mechanisms for the deletions²⁷ and has suggested hypotheses for explaining the different phenotypes observed, particularly in the case of the higher Hb F production that is observed in HPFH relative to $\delta\beta$ -thalassemia.^{13,14,16,28,29} Spanish $\delta\beta^0$ -thalassemia has been very important in these studies; its 5' breakpoint¹³ is within a few hundred nucleotides of the 5' breakpoints of two different HPFH,^{14,24} while the 3' end of the deletion lies beyond the endpoints of all other cases described.²⁴ The definition of the 3' endpoint of Spanish $\delta\beta^0$ -thalassemia, as well as of the DNA sequences specifically deleted in this condition is a necessary preliminary condition to experiments seeking the explanation, according to the above-mentioned models, of differences between HPFH and $\delta\beta$ -thalassemia.

We have cloned a unique DNA fragment from the 3' endpoint of Spanish $\delta\beta^0$ -thalassemia, that appears to lie approximately 11 and 17 kb 3' of the endpoints of black HPFH 1 and 2, respectively.¹⁶

MATERIALS AND METHODS

DNA was obtained from peripheral blood leukocytes of a patient homozygous for Spanish $\delta\beta^0$ -thalassemia.^{13,15} A *Bam* HI digest was enriched in fragments approximately 15 kb in size by centrifugation on a 5% to 20% NaCl gradient and the DNA ligated into EMBL 4 *Bam* HI digested arms and packaged in vitro. After screening with a $\Psi\beta$ -globin probe (the 4.2 kb *Bgl* II fragment), two identical phages containing the abnormal 15.5 kb fragment were obtained, digested with *Hind* III, and subcloned into pBR322. Two unique sequences were prepared: a *Hind* III/*Bam* HI 0.3 kb fragment and a *Hind* III/*Xba* I 1.3 kb fragment to be used as probes in genomic mapping (Fig 1). A 7.6 probe is an *Eco* RI 1.0 kb fragment derived from a clone containing the 3' breakpoint of black HPFH type 1 (kindly provided by Dr B. Forget).¹⁶ Restriction enzyme digestion, hybridization, and washing procedures were as previously described.¹⁵

RESULTS

The 5' breakpoint of the deletion in Spanish $\delta\beta^0$ -thalassemia has been located between the $\Lambda\gamma$ and δ -globin genes immediately 3' to the RIH probe^{13,15} (Fig 1). Using a $\Psi\beta$ -globin fragment as a probe we cloned a 15.5 kb *Bam* HI fragment encompassing the breakpoint, from which we generated the unique DNA *Hind* III/*Bam* HI 0.3 kb and *Hind* III/*Xba* I 1.3 kb probes shown in Fig 1. Fine restriction enzyme mapping of the clone together with genomic mapping using both probes allow us to define in detail the region surrounding the breakpoint both in Spanish $\delta\beta^0$ -thalassemia and in normal DNA (Figs 1 and 2). Four different restriction enzyme sites (*Bam* HI, *Pst* I, *Hind* III, and *Bgl* II) located 5' to the 0.3 kb probe in normal DNA appear to correspond as to their relative order and distance to restriction sites located at the 3' end of a fragment previously cloned from the 3' endpoint of black HPFH 1.¹⁶ The positions of *Hind* III and *Bgl* II sites, both located 3' to the breakpoint, have been defined precisely by direct analysis of our $\delta\beta^0$ -thalassemic clone and confirmed in normal DNA by genomic blotting (not shown). The positions in normal DNA of the *Bam* HI

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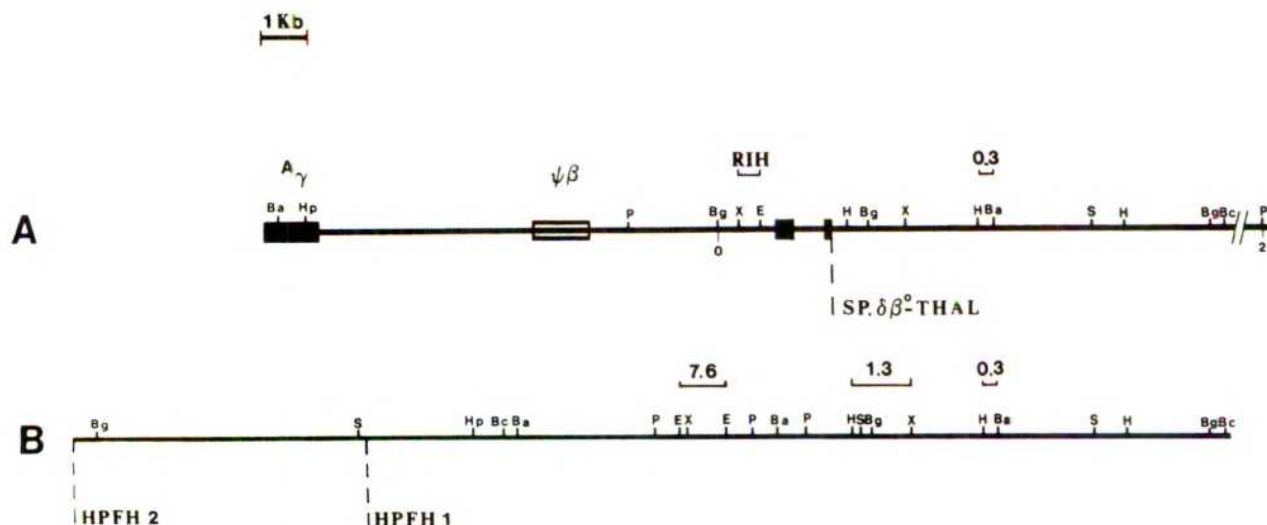


Fig 1. (A) Restriction map of the breakpoint region of Spanish $\delta\beta^0$ -thalassemia. The breakpoint within the *Alu I* repeat (black rectangle) is indicated by a dotted line. The position of the restriction sites is from refs. 13, 15, 24 and this article. (B) Map of the normal region corresponding to the 3' breakpoint of Spanish $\delta\beta^0$ -thalassemia and black HPFH 1 and 2 (indicated by dotted lines) on the basis of ref. 16 and this article. Probes used in this study are indicated (see text for details) over the DNA line. Abbreviations: Ba, *Bam* HI; Hp, *Hpa* I; P, *Pst* I; Bg, *Bgl* II; X, *Xba* I; E, *Eco* RI; H, *Hind* III; Bc, *Bcl* I; S, *Sac* I.

and *Pst* I sites, which lie 5' to the Spanish $\delta\beta^0$ -thalassemia 3' breakpoint, have been defined by genomic mapping: a *Bam* HI/*Pst* I double digestion was used and demonstrated the shortening of the *Bam* HI fragment (Fig 2). These data suggest that HPFH 1 and Spanish $\delta\beta^0$ -thalassemia breakpoints may lie close together. Two additional digestions prove this point: *Bcl* I digestion of normal DNA gives an ~16 kb fragment that appears to hybridize both to the probe 7.6 (derived from the clone containing the black HPFH 3' breakpoint)¹⁶ and to a *Hind* III/*Xba* I 1.3 kb fragment (from the Spanish $\delta\beta^0$ -thalassemic clone) (Fig 2). Similarly, both the 7.6 and the 1.3 kb *Hind* III/*Xba* I probe hybridize to the same 5 kb *Xba* I fragment (Fig 2).

DISCUSSION

Deletions involving the human δ and β globin genes are usually associated (with the exception of that causing Hb Lepore) with substantial increases in the expression during the adult period of the G γ and A γ globin genes adjacent to the deletion. G γ A γ ($\delta\beta$) thalassemias are characterized, in the heterozygous state, by a 20- to 40-fold increase of Hb F levels, relative to normal adults; on the other hand, HPFHs show a further two- to threefold increase, up to ~30% Hb F. The reasons for the higher Hb F levels observed in HPFH patients remain hypothetical; as the breakpoints of the deletions occurring in these syndromes are spread over at least 100 kb, it is difficult to compare different deletions and

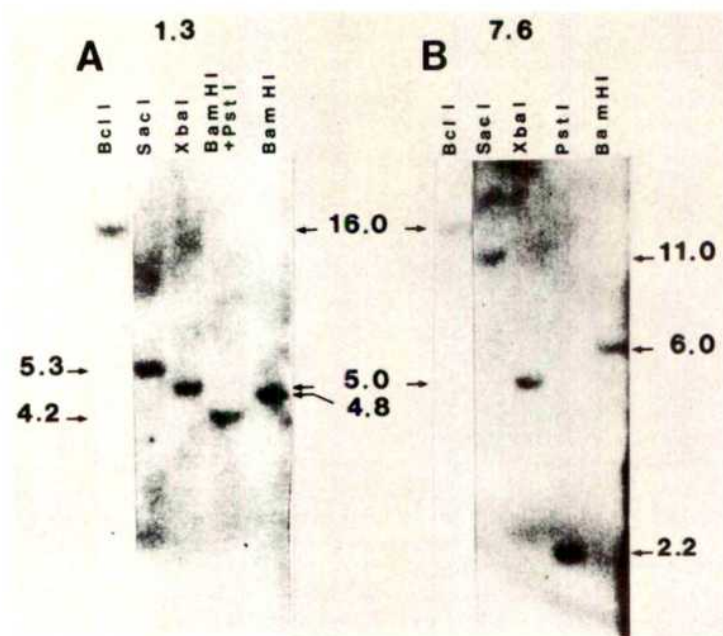


Fig 2. Restriction fragments obtained in a normal DNA with the *Hind* III/*Xba* I 1.3 kb probe prepared from Spanish $\delta\beta^0$ -thalassemia clone (A) and the 7.6 probe from the 3' region of black HPFH 1¹⁶ (B). Numbers indicate the size of the fragments in kb. Both probes detect comigrating bands in *Bcl* I and *Xba* I digests (lanes 1 and 3). The correct position of the normal *Pst* I site 5' to the 3' breakpoint of Spanish $\delta\beta^0$ -thalassemia is established by the shortening of the *Bam* HI/*Pst* I double digest (A: lane 4) in respect to the *Bam* HI fragment (A: lane 5) detected by the 1.3 probe.

to provide a unifying explanation for the various phenotypes (summarized in refs. 24 and 30). Spanish $\delta\beta^0$ -thalassemia is interesting from this point of view, as its 5' breakpoint occurs few hundred base pairs 3' of the 5' breakpoints of two independent HPFH (Italian HPFH and black HPFH type 1).^{14,24} It has been suggested that differences in the extent of the deletion in this region might be responsible for determining the HPFH rather than the $\delta\beta$ -thalassemia phenotype.^{13,14,23,24} On the other hand, it was also proposed that enhancer-like sequences might be present in the region 3' of the HPFH 3' breakpoints and be brought by the deletion into the γ -globin gene region, thereby stimulating its expression.^{16,29} Based on this idea the thalassemia phenotype (in the Spanish case) could be due to the deletion of these enhancer-like sequences.^{24,29} The precise definition of the 3' DNA region that is lost in Spanish $\delta\beta^0$ -thalassemia is crucial to this point; our present data establish the boundaries of the region (approximately 10 kb long) that is expected to include the hypothetical enhancer sequence. Both the 5' and 3' regions where differences between black HPFH 1 and 2 and Spanish $\delta\beta^0$ -thalassemia occur, are now cloned^{13,14,16}; this should allow systematic attempts at defining the roles, if any, of these sequences in the pathological regulation of γ globin expression. Preliminary data²⁹ suggest the existence of a region with some properties of an enhancer approximately 2 kb 3' to the HPFH 1 3' breakpoint.

5' and 3' breakpoints of the deletions in the non- α -globin cluster are spread over at least 200 kb^{5,16}; with more and

more deletions being defined, it is increasingly apparent that breakpoints may not be random. Several HPFH 5' breakpoints occur within 7 kb of the Spanish $\delta\beta^0$ -thalassemia 5' breakpoint; two possible clusters of 3' breakpoints are suggested by the deletion endpoints of Indian³⁰ and Italian HPFH,²⁴ and also of Chinese $G\gamma(A\gamma\delta\beta)$ -thalassemia,²³ Spanish $\delta\beta^0$ -thalassemia, and black HPFH 1 and 2.¹⁶ These data support the idea that specific DNA loops might be intermediates in the generation of these deletions.²⁷ A mechanism involving the joining of distant specific sequences to form a double stem-loop structure, possibly predisposing to the deletion was recently suggested for deletions in the LDL-receptor gene and in our $\delta\beta$ -thalassemia case.³¹

The sequences joined together by the deletion in the LDL-receptor gene originally lie at a distance of approximately 5 kb only; in our case the distance between the joined sequences is at least 90 kb (possibly more than 130 kb according to the recent determination of the extent of the deletion in HPFH 1 by pulsed field gel electrophoresis³²). This fact is also in agreement with the idea that specific mechanisms may be required to bring together distant sequences in the course of processes generating the deletion.³⁰

Availability of the normal DNA and of the rearranged DNA surrounding the breakpoint should allow to confirm the proposed mechanism³¹ and to test for the possible introduction in the course of the rearrangement event of extraneous DNA sequences.²⁸

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CONCISE REPORT

Deposition of Autofluorescent Eosinophil Granules in Pathologic Bone Marrow Biopsies

By Michael K. Samoszuk and Froilan P. Espinoza

Eosinophil granules are intensely autofluorescent when excited by green light. To determine if eosinophils degranulate in the bone marrows of patients with a variety of diseases, we used green light epifluorescence microscopy to examine deparaffinized and dekenkerized sections of 49 bone marrow core biopsies. In 14 of the biopsies, there was striking extracellular deposition of intensely autofluorescent eosinophil granules in addition to numerous intact eosinophils. Among the 14 specimens with extracellular autofluorescence were seven cases of leukemia, four cases of non-Hodgkin's lymphoma, two cases of myelofibrosis,

and one case of pancytopenia with eosinophilia. In the remaining 35 specimens, only intact eosinophils were identifiable. There was no extracellular autofluorescence in three normal marrows, four marrows from AIDS patients, or three biopsies from patients with idiopathic thrombocytopenic purpura (ITP). We conclude that green light epifluorescence microscopy identifies extracellular deposits of eosinophil granules in bone marrow biopsies of some neoplastic disorders and in diseases associated with reticulin fibrosis.

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IN BONE MARROW and peripheral blood, the granules of unstained human eosinophils are strikingly autofluorescent.^{1,2} The intensity and specificity of the autofluorescence allow eosinophils to be easily distinguished from other leukocytes by fluorescence microscopy or by cell-sorting.²

Recent immunocytochemical studies indicate that eosinophils release granule proteins in soft tissues of patients with a variety of diseases.³⁻⁵ By contrast, eosinophil degranulation in bone marrow biopsies has been difficult to document because of the tissue denaturation produced by acidic fixation and decalcification.

In order to determine if eosinophil degranulation occurs in the bone marrows of patients with a variety of diseases, we examined unstained sections of deparaffinized and dekenkerized bone marrow core biopsies by epifluorescence microscopy. This report, therefore, compares the autofluorescence patterns of normal bone marrows, bone marrows with eosinophilia, and bone marrows from patients with neoplastic or myeloproliferative disorders.

MATERIALS AND METHODS

Tissues. Cases for study were retrieved from the Hematology Division files (1985-1987) at UCI Medical Center. Except as noted, only initial diagnostic or staging bone marrow biopsies of high quality (well-fixed; without crush artifact) were studied. The cases were grouped into the diagnostic categories listed in Table 1. For comparative purposes, we also studied three normal bone marrow biopsies and ten biopsies of patients with marked marrow eosinophilia (Hodgkin's disease, three cases; AIDS, four cases; idiopathic thrombocytopenic purpura [ITP], three cases).

All patients were biopsied in the course of a diagnostic workup and were advised of the nature of the procedure and its attendant risks, in accordance with The University of California guidelines. The patients gave informed consent to the procedure and agreed that the tissue may be used for research, at the discretion of the pathologist. Because the study used only archival pathology tissue, it was reviewed and exempted from further approvals by the UCI Human Studies Review Committee.

The Zenkers-fixed, paraffin-embedded bone marrow biopsies were sectioned at 6- μ m thickness, deparaffinized in xylene and graded alcohols, and dekenkerized in Lugol's iodine solution. The sections were then coverslipped with PBS-glycerol (low-fluorescence) and examined within 24 hours.

Epifluorescence microscopy. The epifluorescent microscopy was performed on a Nikon Optiphot microscope (Nikon Inc, Garden

City, NY) equipped with an EF-D episcopic-fluorescence attachment, UFX-II camera, and planapochromat objectives. A green light excitation filter (546 nm) was used together with a DM580 dichroic mirror and 580W eyepiece-side barrier filter. Illumination was provided by a high pressure mercury lamp (HBO100W/2).

Sections were examined independently by two observers, and the presence or absence of extracellular autofluorescent granules was recorded. For photomicrography, Kodak professional TMAX black and white film (ASA 400) was used.

RESULTS

Epifluorescence microscopy. The results of the visual examination of 49 cases are summarized in Table 1. In all instances, intact eosinophils were easily visualized (Fig 1); and in 14 of the cases, there was extracellular deposition of autofluorescent eosinophil granules. Except in two cases believed to contain coarse globules of slightly fluorescent lipofuscin, there was agreement between both observers on the presence or absence of extracellular autofluorescence. Older specimens had less intense fluorescence than more recent biopsies.

The extracellular autofluorescence was particularly extensive in the biopsies of chronic myelogenous leukemia (Fig 2). There was no consistent microanatomic localization of the granules; in some cases, a fine granularity was present in a patchy distribution while in others a paratrabecular deposition was noted. Extracellular deposits of autofluorescent granules were not identified by any of the normal bone

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Table 1. Results of Epifluorescent Microscopy of Bone Marrows

Diagnosis	Eosinophil Degranulation	
	Absent	Present
Normal bone marrow (3)	3	0
Eosinophilic bone marrow in patients with Hodgkin's disease (3)	3	0
Eosinophilic bone marrow in patients with AIDS (4)	4	0
Idiopathic thrombocytopenic purpura and marrow eosinophilia (3)	3	0
Reticulin fibrosis; pancytopenia (1)	0	1
Marrow eosinophilia; pancytopenia (1)	0	1
Chronic myelogenous leukemia (3)	1	2
Acute myelogenous leukemia (10)	8	2
Acute myelomonocytic leukemia (1)	0	1
Acute lymphocytic leukemia (11)	9	2*
Myelofibrosis (2)	1	1
Non-Hodgkin's lymphoma in marrow (7)	3	4*
Totals	35	14

*Including one case after chemotherapy. Extracellular deposits of a faintly fluorescent material were interpreted to be lipofuscin by one observer.

marrows or in the hypereosinophilic biopsies from patients with Hodgkin's disease, AIDS, or ITP.

DISCUSSION

The autofluorescence of 49 bone marrow biopsies representing a variety of diseases was studied. Extracellular

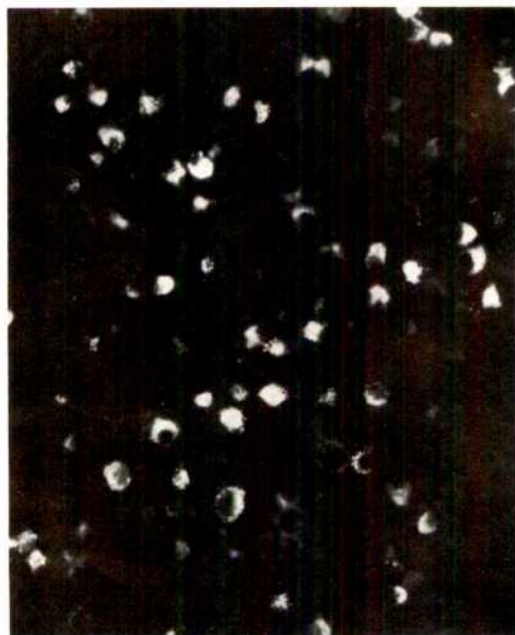


Fig 1. Photomicrograph of a bone marrow with eosinophilia but no evidence of extracellular autofluorescence. Intact eosinophils are brightly fluorescent and contain discrete cytoplasmic granules. Green light epifluorescence, with no counterstain. (Original magnification $\times 400$; current magnification $\times 304$.)



Fig 2. Photomicrograph of a bone marrow from a patient with chronic myelogenous leukemia. In addition to the numerous intact eosinophils, there is abundant deposition of finely granular autofluorescent material between the bone marrow cells. No counterstain. (Original magnification $\times 400$; current magnification $\times 304$.)

deposition of autofluorescent granules was observed only in neoplastic conditions and in diseases associated with reticulin fibrosis, suggesting that the phenomenon is not an artifact of specimen processing. Because autofluorescence in marrows is relatively specific for eosinophil granules, we conclude that green light epifluorescence microscopy detects eosinophil degranulation in the bone marrow of patients with a variety of disorders.

The biological significance of this observation is not yet known, but the implications are intriguing. Eosinophil degranulation has been described in a number of disorders, including atopic dermatitis,³ onchocerciasis,³ eosinophilic endomyocardial disease,⁴ and Hodgkin's lymphomas.^{5,6} All of these diseases are associated with some degree of fibrosis, and in this study, a similar association is suggested.

Moreover, the eosinophil granule is known to contain a wide variety of compounds with potent biological activities.⁷ We therefore speculate that degranulation of eosinophils in bone marrows with neoplasms could conceivably modulate the host inflammatory response to the neoplasm. Future studies correlating eosinophil degranulation with the clinical course will be of interest in determining the biological significance of the degranulation phenomenon in bone marrows.

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CORRESPONDENCE

PROPLATELETS AND STRESS PLATELETS

To the Editor:

Recently, Penington and his colleagues presented evidence to support their hypothesis that megakaryocyte fragments or proplatelets may contribute to the macrothrombocytosis that occurs in situations of altered platelet production.¹ They measured platelet count with K_2 EDTA as anticoagulant and mean platelet volume (MPV) with ACDE₁ as anticoagulant, in blood harvested from rats after a single injection of antiplatelet serum. Twenty-four hours after the injection, the animals were acutely thrombocytopenic and the MPV was significantly raised above control values. At this time, platelet rich plasma (PRP) was observed in the phase-contrast microscope. In blood taken into ACDE₁ from controls, ~6% of the platelets were nondiscoid and were classified as proplatelets. In thrombocytopenia, this value increased to ~16% to 18%. There was no significant difference in the number of proplatelets in central venous or arterial blood in either thrombocytopenic or control animals. In contrast, only discoid platelets could be observed in PRP derived from both thrombocytopenic and control blood anticoagulated with K_2 EDTA. Hence, it was argued that proplatelets in the blood of thrombocytopenic animals elevated the MPV. Their absence with K_2 EDTA was explained by the shape change induced by this anticoagulant.

These observations raise some fundamental issues. Cells observed under the phase contrast microscope may not be "sensed" in the same way by an aperture impedance system. As a single cell passes through the aperture, it changes the impedance between two electrodes. The height of the voltage pulse is calibrated via an analogue-to-digital convertor (ADC). Usually, the ADC has a facility for editing pulses with an abnormal shape or width.²

In this system, the proplatelets shown in Figs 5B, 6, and 7 in ref. 1 are indistinguishable from coincident platelets. The associated pulses will be abnormal and edited out. Hence, they will not contribute to the measured MPV. In particular, the branching proplatelet displayed in Fig 7 is ~50 μ m in length, the same size as the aperture of the Coulter ZF.¹

The channel number of 2,000 quoted by Tong and colleagues¹ suggests that they used a 10-bit ADC. No indication was given of the volume range that could be measured. (Current commercial systems use a 6-bit ADC giving 64 channels over a volume range of 2 to 20 fL for platelets.²) If the upper threshold of volume was less than the size of the large cytoplasmic fragments, then, irrespective of shape, these volumes would not contribute to the measured MPV.

Using an aperture impedance system with an 8-bit ADC giving 256 channels over 0 to 25 fL, we observed that the major changes in platelet volume occurred in the first 12 hours after a single APS injection.³ By 24 hours, the increase in MPV above control values was comparable with that reported by Tong and colleagues.¹ Neither the thrombocytopenic nor control rats had a log Gaussian platelet volume distribution.³ There was no evidence of platelet agglutination or aggregation under the phase-contrast microscope in blood taken into citrate and prostaglandin E₁.

Tong and colleagues¹ proposed that proplatelet chains break into fragments of lesser length, possibly within the microcirculation, when they are caught astride bifurcating vessels. If this were so and the observed proplatelets in ACDE₁ are not artifactual, it is surprising that there is no difference between the percentage of proplatelets in inferior vena cava and aortic blood. After blood has passed through the pulmonary microcirculation, a reduction in proplatelets might be expected from this production mechanism.

Finally, the presence of bare megakaryocyte nuclei in the bone marrow does not necessarily indicate that production of proplatelets in marrow sinusoids must occur. The cytoplasm can stream away from the nucleus as an intact unit, leaving the naked nucleus behind in the marrow.⁴

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To the Editor:

Trowbridge rightly notes that an aperture impedance system and pulse-height analyzer may not fully and accurately measure the volume of nonspherical particles when used for determination of platelet volume in blood anticoagulated with acid-citrate-dextrose (ACD). Indeed, it will underestimate platelet volume under these circumstances, as elongated proplatelets, such as those illustrated in our article, are likely to flow through the aperture along their long axis. That they are not detected by such a system does not mean that

they are not present, but rather that they are likely to make an even greater contribution to macrothrombocytosis in EDTA-anticoagulated blood than might otherwise have been thought. Proplatelets in marrow sinusoids¹ or in blood² depend on microtubules for their elongated form. Depolymerization by EDTA will inevitably alter their shape into spherical form.

The pulse-height analysis system used in our study used 512 channels and detected platelets of volume from 1 to 34 fL. Our system has no editing circuits to remove pulses of abnormal shape or width. It is of interest that Trowbridge and colleagues³ noted

comparable increase in platelet volume at 24 hours in their acutely thrombocytopenic rats, and we agree that an even more striking increase in volume may occur earlier than this, as noted in 1976 by Odell and associates,⁴ long before any change in megakaryocyte DNA could occur—previously suggested as the principal regulator of platelet volume.⁵

Trowbridge should not be surprised at the finding of proplatelets in aortic blood. He and his colleagues have argued for substantial production of platelets from megakaryocytes lodged in the pulmo-

nary circulation.⁶ These will contribute proplatelets to the arterial circulation as will those from marrow sinusoids to blood in the IVC.

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ANNOUNCEMENTS

AMERICAN SOCIETY OF HEMATOLOGY TWENTY-NINTH ANNUAL MEETING

December 5-8, 1987

Sheraton Washington/Omni Shoreham Hotels
Washington, DC

The Twenty-Ninth Annual Meeting of the American Society of Hematology will be held at the Sheraton Washington and Omni Shoreham Hotels, Washington, DC, December 5-8, 1987. Programs by the Education Committee and the Scientific Subcommittees will be held on Saturday, December 5, and Sunday, December 6. The Presidential Symposium will be Tuesday morning, December 8. Four mini-plenary sessions will be held on Tuesday afternoon. Selected papers submitted to the meeting will be presented either in oral or poster sessions on Sunday, December 6; Monday, December 7; and Tuesday, December 8.

The abstract deadline for contributed papers is September 1, 1987. Abstract forms may be obtained from SLACK, Inc (address below). Subsequent issues of *BLOOD* will contain details of the Educational and Subcommittee Programs and the Presidential Symposium, in addition to information about registration for the meeting. Preregistration material will be sent early this fall to members. If you do not receive your meeting information by October 6, 1987, please contact the Registration Supervisor at SLACK, Inc, 6900 Grove Rd, Thorofare, NJ 08086-9447; (609) 848-1000. Nonmembers wishing to receive preregistration information and forms should also contact SLACK, Inc.

AMERICAN SOCIETY OF HEMATOLOGY 29TH ANNUAL MEETING

December 5-8, 1987

ADVANCE REGISTRATION

NAME _____ STREET _____

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American Society of Hematology Members, \$30.00 (A) _____ Honorary or Emeritus Members, no fee (B) _____ Council on Thrombosis Members, \$30.00 (C) _____ Nonmembers, \$55.00 (D) _____ *Nonmembers in Training, \$10.00 (E) _____ Medical Technologists, \$40.00 (F) _____ Spouse, \$10.00 (G) _____

Registration includes one copy of the abstract booklet (supplement to *Blood*) and one copy of the Education Program. Additional copies desired _____ (indicate number desired). Supplement to *Blood* \$20.00 _____ Education Program \$6.00 _____

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Mail this form and check to: ASH Business Office, Registration Supervisor, SLACK, Inc, Box 510, Thorofare, NJ 08086-0510. **Advance registration deadline is November 2, 1987. All registrants who fail to register by November 2, 1987, will be charged an additional fee of \$10.00 upon registration at the Annual Meeting.** (Checks payable to the American Society of Hematology must accompany registration.) Request for refunds must be submitted in writing by December 8, 1987.

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Upon receipt of this Advance Registration, housing forms and additional meeting and social information will be mailed to you.

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The University of Rochester School of Medicine offers research training to physicians who have completed at least 1 year of residency or research experience. Fellowships will begin July 1, 1988. Opportunities exist in any discipline relevant to the cause, diagnosis, treatment, or prevention of cancer, including endocrinology, experimental therapeutics, genetics, hematology, immunology, microbiology, molecular and cellular biology, pathology, and radiation oncology.

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For further information, contact: Wilmot Cancer Research Fellowship Program, Medical Center Box 601, University of Rochester School of Medicine & Dentistry, 601 Elmwood Ave, Rochester, NY 14642.

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For further information, contact: Stephen E. Jones, MD, Charles A. Sammons Cancer Center, Room 4800, Baylor University Medical Center, 3500 Gaston Ave, Dallas, TX 75246. Phone: (214) 820-2021.

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The AAFA has established a Research Fellowship Award in order to encourage young researchers and to recognize their contributions to research related to aplastic anemia. In January 1988, an award of \$25,000 will be made for a research fellowship to begin July 1988. The deadline for completion of the application package is November 15, 1987.

For information concerning eligibility criteria and application forms, write: Research Fellowship Award, AAFA, PO Box 22689, Baltimore, MD 21203.

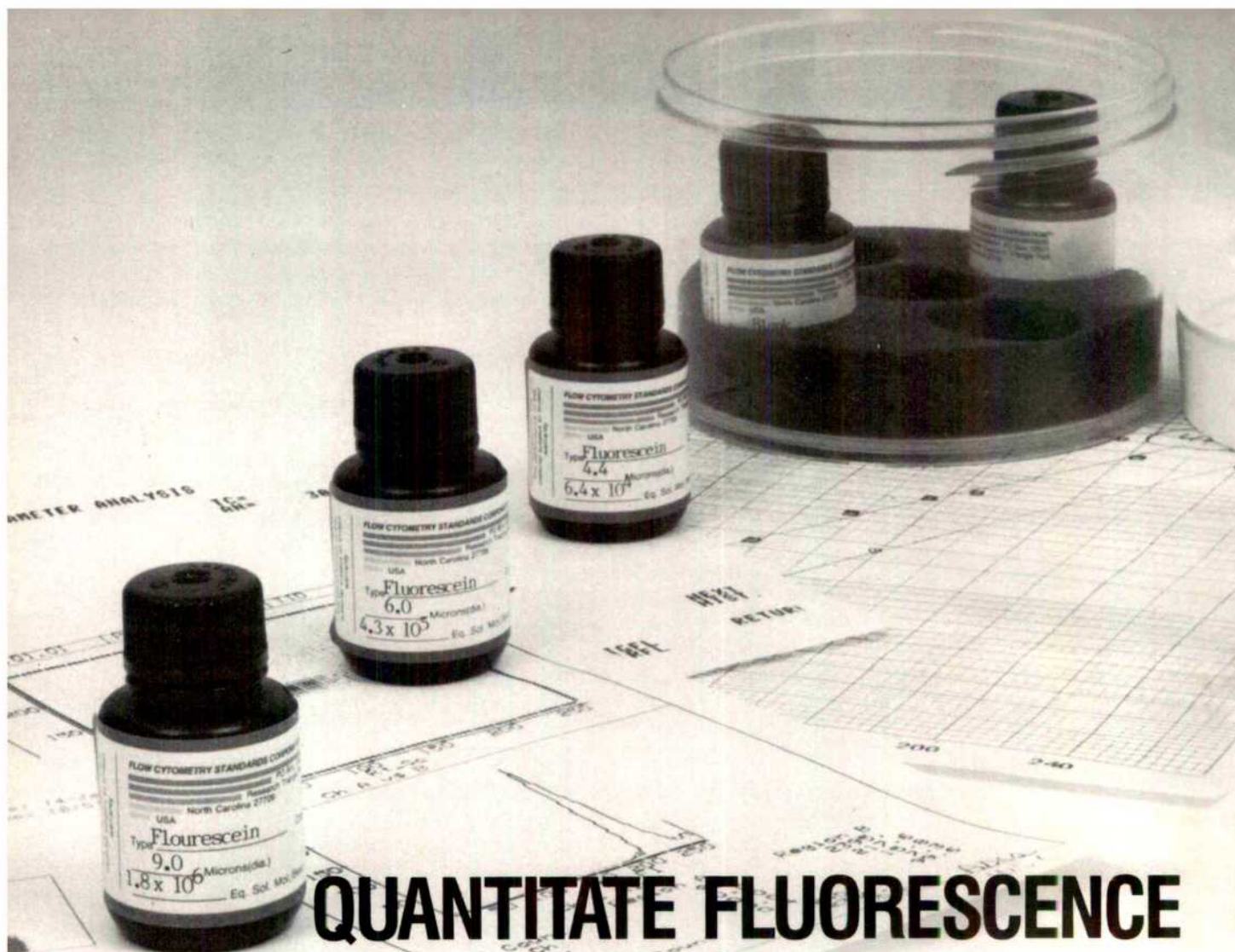
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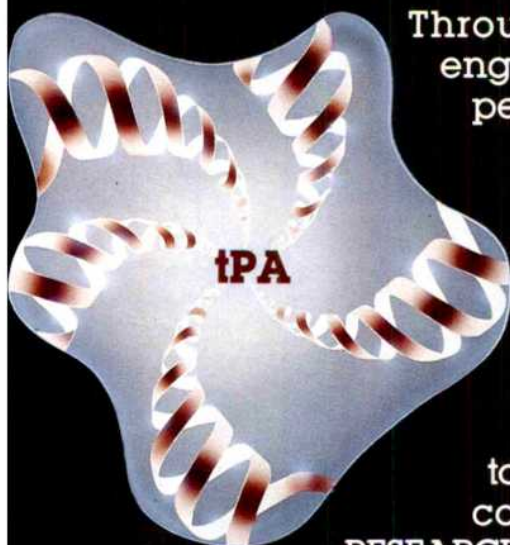
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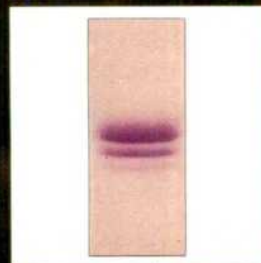
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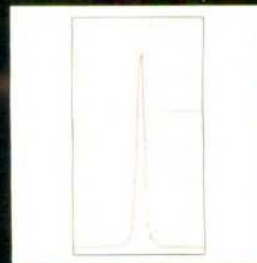
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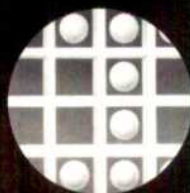
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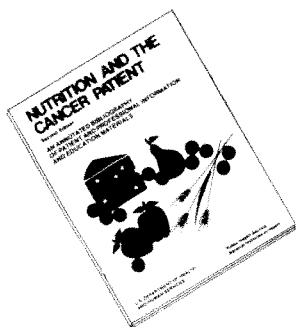
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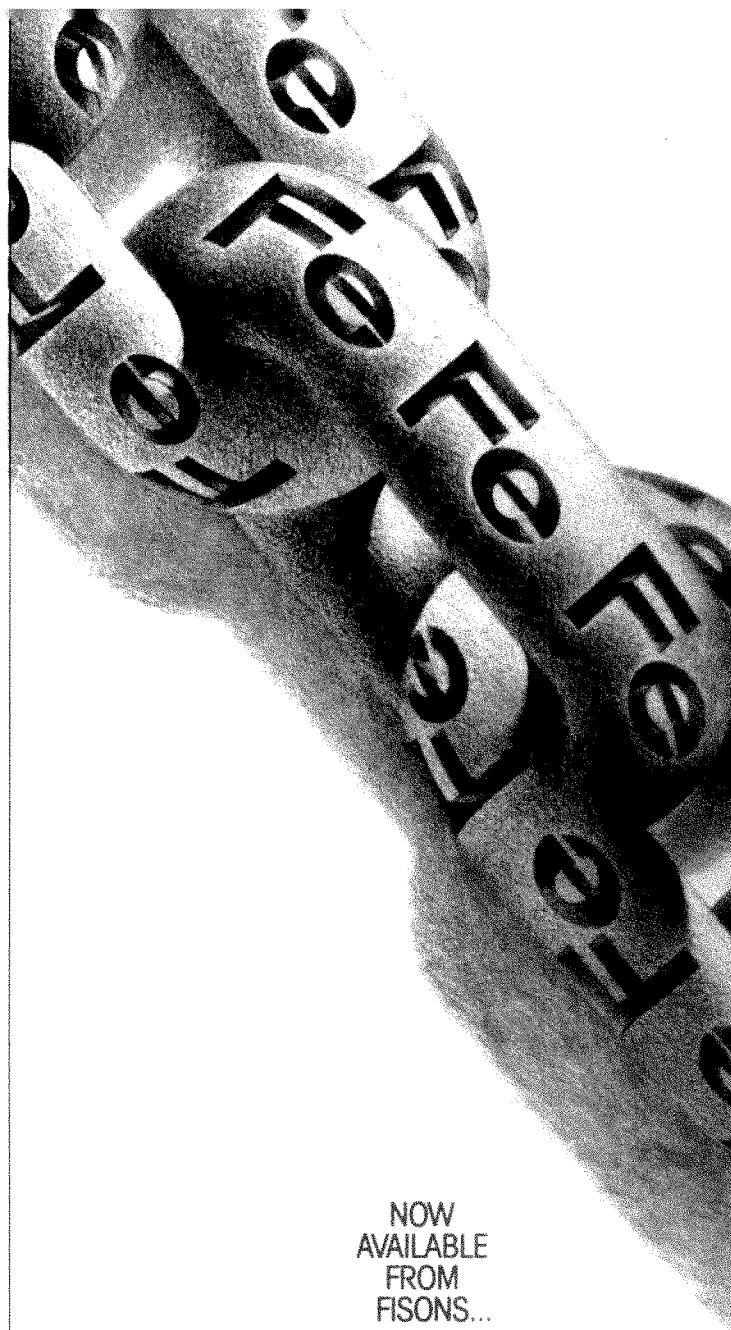
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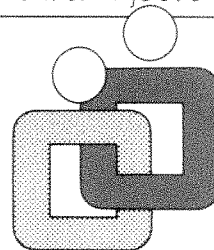


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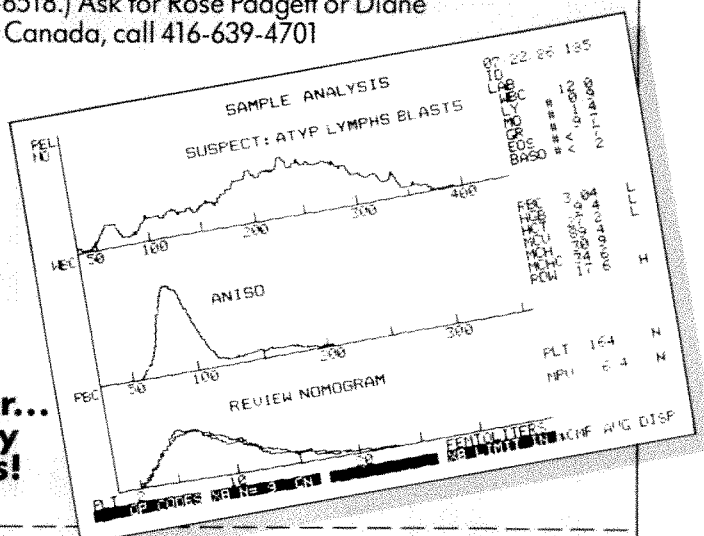
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Abdominal/Pelvic CT
February 25



Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al*: Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al*: Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al*: Combination chemotherapy advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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M-BACOD

4. Skarin AT, Canellos GP, Rosenthal DS, *et al*: Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, *et al*: Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al*: Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), m-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

UA-154-1

Blenoxane[®] (sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane[®] be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonia.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI_{O_2} at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban[®]. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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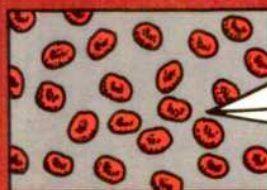
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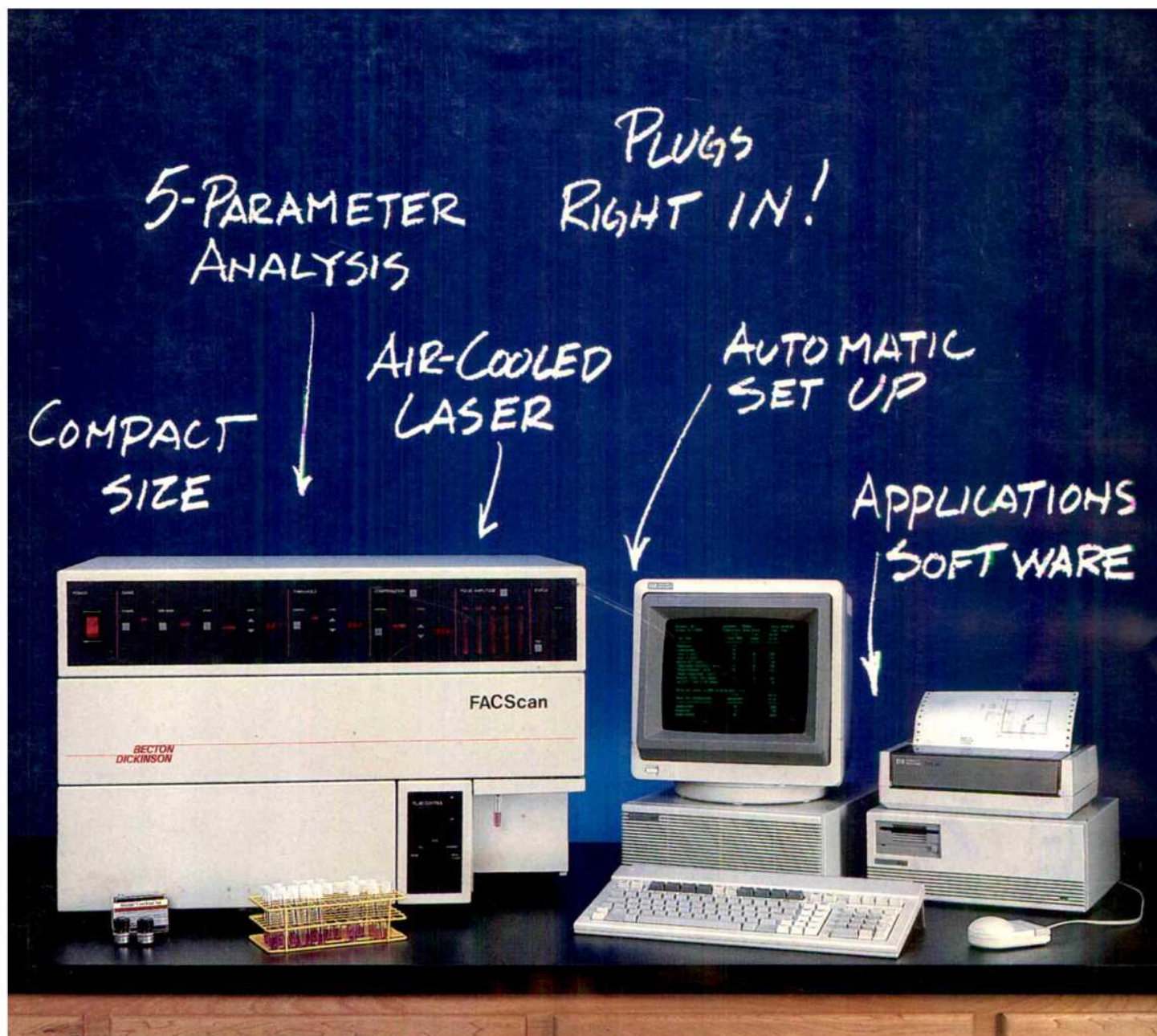
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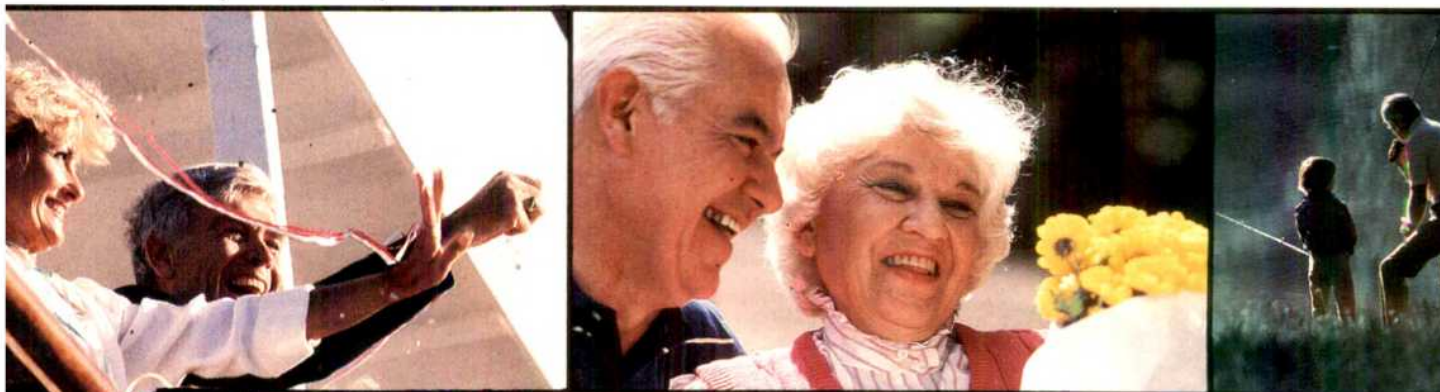
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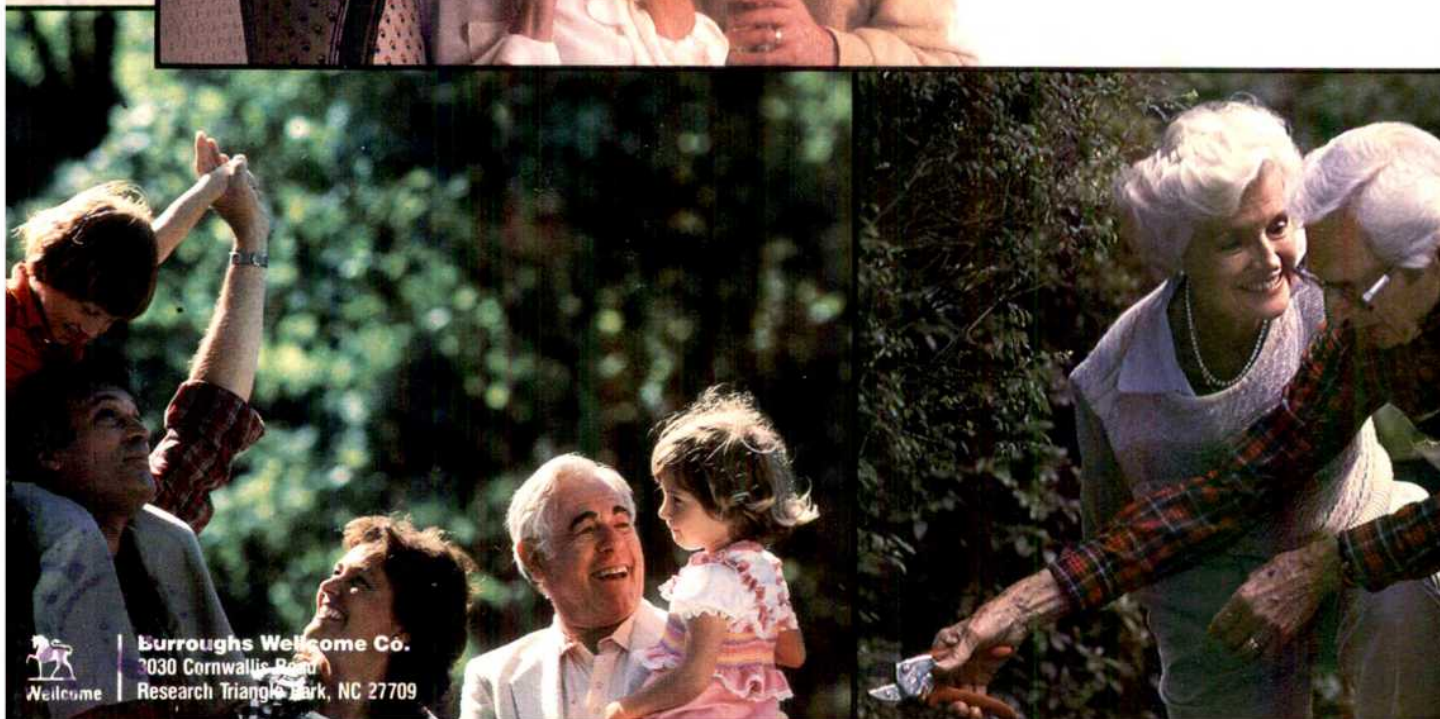
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CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B₁₂. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m² orally or parenterally followed by 10 mg/m² orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m² every three hours until the serum methotrexate level is below $5 \times 10^{-8} M$.^{1,2}

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.^{3,4,5}

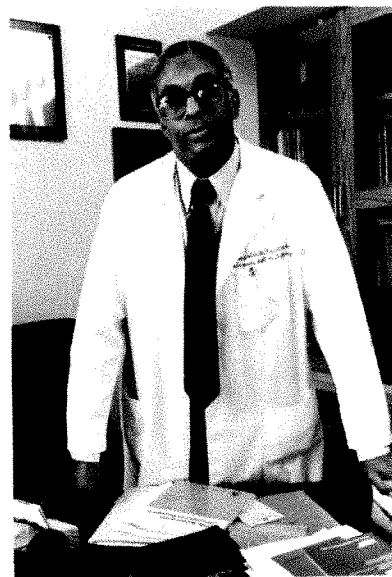
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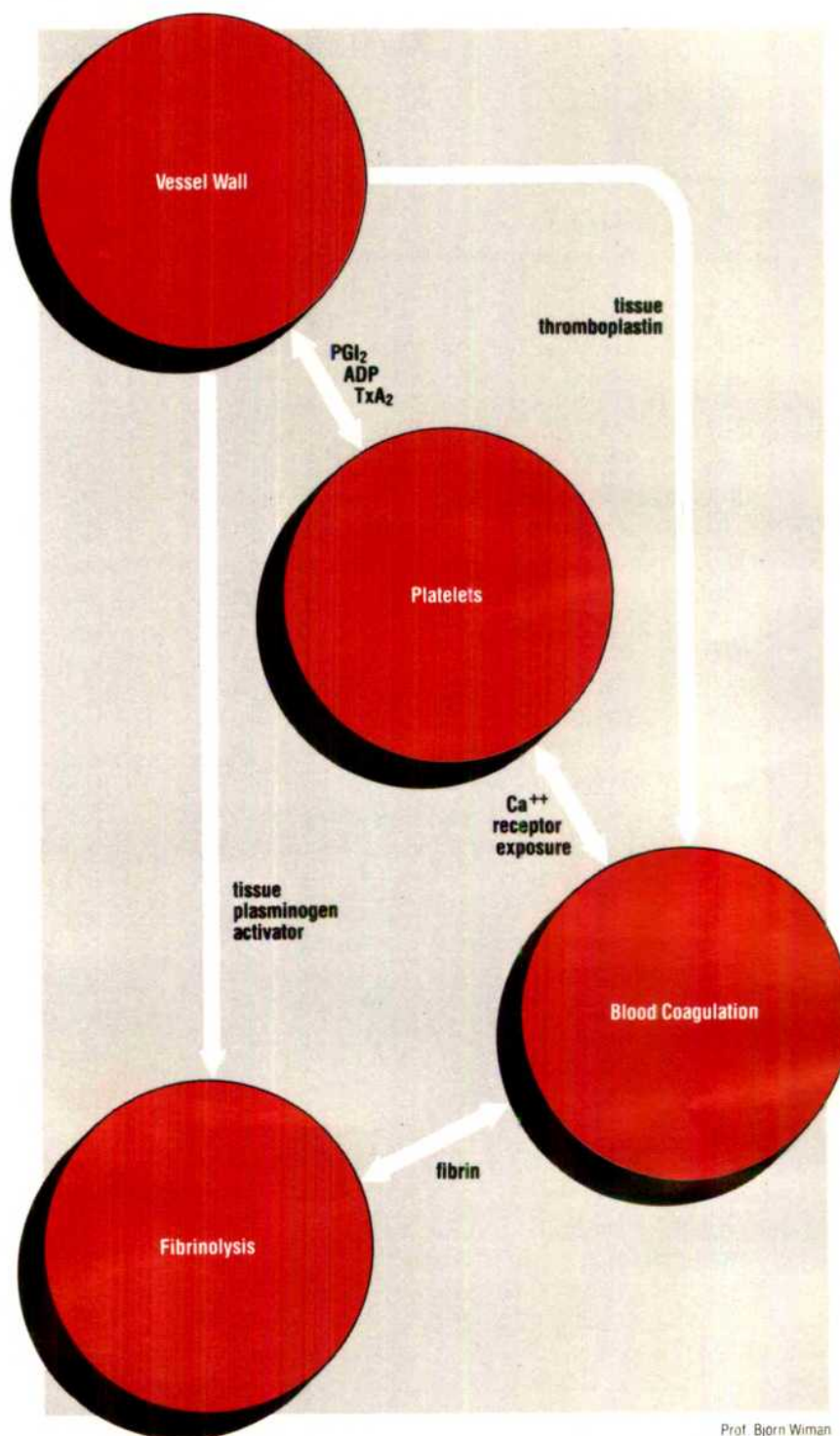
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*The Journal of
The American Society of Hematology*

VOL 70, NO 3

SEPTEMBER 1987

CONTENTS

Review: Structure-Function Relationship of Human Von Willebrand Factor	<i>Jean-Pierre Girma, Dominique Meyer, Cornelis L. Verweij, Hans Pannekoek, and Jan J. Sixma</i>	605
Bipotential Cell Differentiation of KU-812: Evidence of a Hybrid Cell Line That Differentiates Into Basophils and Macrophage-Like Cells	<i>Takeaoki Fukuda, Kenji Kishi, Yoshihisa Ohnishi, and Akira Shibata</i>	612
Patients With a Prolonged Bleeding Time and Normal Aggregation Tests May Have Storage Pool Deficiency: Studies on One Hundred Six Patients	<i>H. Karel Nieuwenhuis, Jan-Willem N. Akkerman, and Jan J. Sixma</i>	620
High Serum Interleukin-2 Receptor Levels Are Related to Advanced Disease and a Poor Outcome in Childhood Non-Hodgkin's Lymphoma	<i>Ching-Hon Pui, Stephen H. Ip, Patrick Kung, Richard K. Dodge, Costan W. Berard, William M. Crist, and Sharon B. Murphy</i>	624
Monoclonal Antibody Ki-B3 Detects a Formalin Resistant Antigen on Normal and Neoplastic B Cells <i>Alfred C. Feller, Hans H. Wacker, Gerhard Moldenhauer, Heinz-J. Radzun, and Mohammad R. Parwaresch</i>		629
T Cell Rearranging Gene γ : Diversity and mRNA Expression in Fresh Cells From T Cell Acute Lymphoblastic Leukemia	<i>Denis Le Paslier, Zhu Chen, Pascale Loiseau, Daniel Cohen, and Francois Sigaux</i>	637
Thromboxane A ₂ Causes Feedback Amplification Involving Extensive Thromboxane A ₂ Formation on Close Contact of Human Platelets in Media With a Low Concentration of Ionized Calcium	<i>Marian A. Packham, Raelene L. Kinlough-Rathbone, and J. Fraser Mustard</i>	647
¹¹¹ In-Oxine Platelet Survivals in Thrombocytopenic Infants	<i>Valerie Castle, Geoffrey Coates, John G. Kelton, and Maureen Andrew</i>	652
The Effects of Three Recombinant Growth Factors, IL-3, GM-CSF, and G-CSF, on the Blast Cells of Acute Myeloblastic Leukemia Maintained in Short-Term Suspension Culture	<i>Jun Miyauchi, Colm A. Kelleher, Yu-Chung Yang, Gordon G. Wong, Steven C. Clark, Mark D. Minden, Salomon Minkin, and Ernest A. McCulloch</i>	657
Immunologic Reconstitution After Haploidentical Bone Marrow Transplantation for Immune Deficiency Disorders: Treatment of Bone Marrow Cells With Monoclonal Antibody CT-2 and Complement	<i>R.C. Moen, S.D. Horowitz, P.M. Sondel, W.R. Borchering, M.E. Trigg, R. Billing, and R. Hong</i>	664
Induction of Proliferation of B Prolymphocytic Leukemia Cells by Phorbol Ester and Native or Recombinant Interferon- γ	<i>R.W. Sauerwein, W.G.J. van der Meer, and L.A. Aarden</i>	670
Analysis of CD1 Molecules on Thymus Cells and Leukemic T Lymphoblasts Identifies Discrete Phenotypes and Reveals That CD1 Intermolecular Complexes Are Observed Only on Normal Cells	<i>Martine Amiot, Helene Dastot, Michel Schmid, Alain Bernard, and Laurence Bousmell</i>	676
Inhibition of Hemopoietic Growth Factor-Induced Proliferation by Adenosine Diphosphate-Ribosylation Inhibitors	<i>Gerardo Colon-Otero, Julianne J. Sando, James L. Sims, Elizabeth McGrath, David E. Jensen, and Peter J. Quesenberry</i>	686
Exposure to γ -Irradiation Increases Phorbol Myristate Acetate-Induced H ₂ O ₂ Production in Human Macrophages	<i>Elaine K. Gallin and Spencer W. Green</i>	694
The Transglutaminase in Vascular Cells and Tissues Could Provide an Alternate Pathway for Fibrin Stabilization	<i>Charles S. Greenberg, Komandoor E. Achyuthan, Michael J. Borowitz, and Marc A. Shuman</i>	702

(Continued on following page)

(Contents continued)

Antiidiotypic IgG Crossreactive With Rh Alloantibodies in Red Cell Autoimmunity	<i>S.P. Masouredis, M.J. Branks, and E.J. Victoria</i>	710
Analysis of Hemoglobin F Production in Saudi Arabian Families With Sickle Cell Anemia	<i>Barbara A. Miller, Mohammed Salameh, Mohammed Ahmed, Nancy Olivieri, Giovanna Antognetti, Stuart H. Orkin, Titus H.J. Huisman, and David G. Nathan</i>	716
Extracellular Matrix of Cultured Bovine Aortic Endothelial Cells Contains Functionally Active Type 1 Plasminogen Activator Inhibitor.....	<i>Jun Mimuro, Raymond R. Schleef, and David J. Loskutoff</i>	721
An α -Globin Gene Initiation Codon Mutation in a Black Family With HbH Disease	<i>Nancy F. Olivieri, Lebe S. Chang, Annette O. Poon, Alan M. Michelson, and Stuart H. Orkin</i>	729
Regulated Expression of Amplified Human β Globin Genes	<i>Deborah Rund, Carl Dobkin, and Arthur Bank</i>	733
Abnormal Stimulated Adherence of Neonatal Granulocytes: Impaired Induction of Surface MAC-1 by Chemotactic Factors or Secretagogues.....	<i>Donald C. Anderson, Katherine L. Becker Freeman, Barbara Heerd, Bonnie J. Hughes, Richard M. Jack, and C. Wayne Smith</i>	740
ADP-Induced Platelet Shape Change and Mobilization of Cytoplasmic Ionized Calcium Are Mediated by Distinct Binding Sites on Platelets: 5'-p-Fluorosulfonylbenzoyladenine Is a Weak Platelet Agonist	<i>A. Koneti Rao and Maria A. Kowalska</i>	751
Defensin-Rich Dense Granules of Human Neutrophils	<i>William G. Rice, Tomas Ganz, Joseph M. Kinkade. Jr, Michael E. Selsted, Robert I. Lehrer, and Richard T. Parmley</i>	757
Molecular Determinants of Clinical Expression of Hereditary Elliptocytosis and Pyropoikilocytosis	<i>Theresa Coetzer, Jack Lawler, Josef T. Prchal, and Jiri Palek</i>	766
Effect of Warfarin on Prothrombin Synthesis and Secretion in Human HEP G2 Cells	<i>Simon Karpatkin, Thomas H. Finlay, Anita L. Ballesteros, and Margaret Karpatkin</i>	773
Long Lasting IgG Subclass and Antibacterial Polysaccharide Antibody Deficiency After Allogeneic Bone Marrow Transplantation	<i>Pierre Aucouturier, Anne Barra, Liliane Intrator, Catherine Cordonnier, Dominique Schulz, Francoise Duarte, Jean-Paul Vernant, and Jean-Louis Preud'homme</i>	779
1986 Update of HIV Seroprevalence, Seroconversion, AIDS Incidence, and Immunologic Correlates of HIV Infection in Patients With Hemophilia A and B.....	<i>Margaret V. Ragni, Alan Winkelstein, Larry Kingsley, Joel A. Spero, and Jessica H. Lewis</i>	786
Biotinylated Erythrocytes: In Vivo Survival and In Vitro Recovery.....	<i>Takashige Suzuki and George L. Dale</i>	791
Direct Evidence for the Interaction of the Nucleotide Affinity Analog 5'-p-Fluorosulfonylbenzoyl Adenosine With a Platelet ADP Receptor	<i>William R. Figures, L. Marie Searce, Patricia DeFeo, Gwendolyn Stewart, Fengxin Zhou, John Chen, James Daniel, Roberta F. Colman, and Robert W. Colman</i>	796
Ca ²⁺ Permeability and Cytosolic Ca ²⁺ Concentration Are Not Impaired In β -Thalassemic and Hemoglobin C Erythrocytes.....	<i>M.D. Rhoda, F. Galacteros, Y. Beuzard, and F. Giraud</i>	804
A δ -Globin Gene Derived From Patients With Homozygous δ^0 -Thalassemia Functions Normally on Transient Expression in Heterologous Cells	<i>Takanori Nakamura, Yoshihiro Takiyama, Yoshiro Ohta, Shigeru Fujita, Yasuyuki Takagi, and Yasuyuki Fukumaki</i>	809
Phenotypic Similarities and Differences Between CALLA-Positive Acute Lymphoblastic Leukemia Cells and Normal Marrow CALLA-Positive B Cell Precursors	<i>Daniel H. Ryan, Charles W. Chapple, Stuart A. Kossover, Avery A. Sandberg, and Harvey J. Cohen</i>	814
Serum Immunoreactive Erythropoietin in Children With Cyanotic and Acyanotic Congenital Heart Disease	<i>Per Hagä, P. Mary Cotes, Janice A. Till, Barbara D. Minty, and Elliot A. Shinebourne</i>	822
Effect of Calcium Ion Concentration on the Ability of Fibrinogen and von Willebrand Factor to Support the ADP-Induced Aggregation of Human Platelets	<i>E.J. Harfenist, M.A. Packham, R.L. Kinlough-Rathbone, M. Cattaneo, and J.F. Mustard</i>	827

(Continued on following page)

(Contents continued)

Selective Uptake of [³ H]Arachidonic Acid Into the Dense Tubular System of Human Platelets	<i>Michael Laposata, Charles M. Krueger, and Jeffrey E. Saffitz</i>	832
Studies With a Monoclonal Antibody Against Activated Platelets: Evidence That a Secreted 53,000-Molecular Weight Lysomone-like Granule Protein Is Exposed on the Surface of Activated Platelets in the Circulation	<i>H. Karel Nieuwenhuis, Joep J.G. van Oosterhout, Erik Rozemuller, Freek van Iwaarden, and Jan J. Sixma</i>	838
The Regulation of Human Factor V by a Neutrophil Protease	<i>Adrian M. Oates and Hatem H. Salem</i>	846
Regulation of Transferrin Receptor Expression in Myeloid Leukemia Cells	<i>Raymond Taetle, Steven Ralph, Shelly Smedsrud, and Ian Trowbridge</i>	852
Neutrophil Elastase Produces 52-kD and 30-kD Glucocorticoid Receptor Fragments in the Cytosol of Human Leukemia Cells	<i>Clark W. Distelhorst, Karen E. Janiga, Kathryn J. Howard, Sarah E. Strandjord, and Edward J. Campbell</i>	860

CONCISE REPORTS

High-Dose Chemoradiotherapy and Autologous Bone Marrow Transplantation for Resistant Multiple Myeloma	<i>Bart Barlogie, Raymond Alexanian, Karel A. Dicke, Gunar Zagars, Gary Spitzer, Sundar Jagannath, and Leonard Horwitz</i>	869
Molecular Analysis of Relapse in Chronic Myeloid Leukemia After Allogeneic Bone Marrow Transplantation	<i>T.S. Ganesan, Gao Ling Min, J.M. Goldman, and B.D. Young</i>	873
Unrelated Donor Bone Marrow Transplantation Therapy for Chronic Myelogenous Leukemia	<i>Philip McGlave, Edward Scott, Norma Ramsay, Diane Arthur, Bruce Blazar, Jeffrey McCullough, and John Kersey</i>	877
Molecular Genetic Rearrangements Distinguish Pre- and Post-Bone Marrow Transplantation Lymphoproliferative Processes	<i>Wesley J. Miller, Ralph S. Shapiro, Rogelio Gonzalez-Sarmiento, and John H. Kersey</i>	882

SPECIAL DEPARTMENTS

Correspondence	886
Announcements	890
Errata	893
Information for Contributors	xxix, xxx

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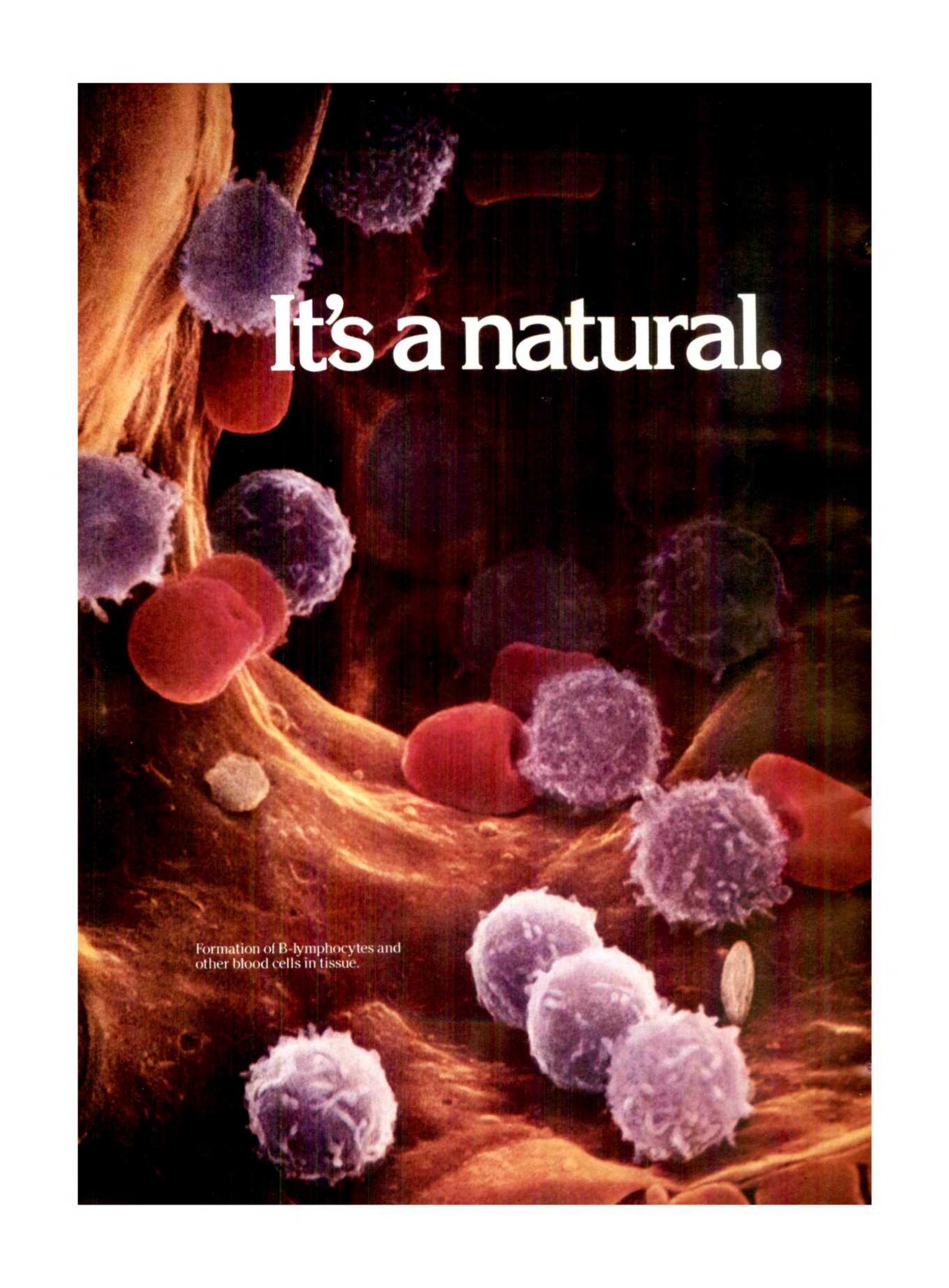


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A scanning electron micrograph showing a cross-section of tissue. Several large, spherical, purple-colored cells with a bumpy, textured surface are visible. These are surrounded by smaller, smooth, reddish-brown disc-shaped cells. The background is a complex, fibrous network of orange and brown structures.

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Gamimune® N contains at least 98% gamma globulin and is the only ready-to-use liquid IGIV available. Cutter's patented fractionation method (based on Cohn-Oncley methods 6 and 9) assures Gamimune® N contains no heterologous proteins, with no detectable levels of protein aggregates or vasoactive enzymes.² And studies show *in vitro* inactivation of HIV (HTLV-III virus)[‡] during production of Gamimune® N.

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*It is not possible to predict which patients with ITP will respond to therapy.

†In patients who respond to therapy, a rise in platelet count is generally rapid (within one to five days), transient (most often lasting from several days to several weeks), and should not be considered curative.

‡The Cohn-Oncley process used to produce Gamimune® N has been challenged *in vitro* with a virus spike of 1×10^5 TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III) and found to remove and/or inactivate the viral challenge. In addition, Gamimune® N undergoes an incubation step as a final container which has been shown to effect an additional 1×10^3 to 1×10^4 reduction TCID (Tissue Culture Infectious Doses) of HIV (HTLV-III).³

1. Gamimune® N Product License Application.

2. Lundblad JL, Mitra G, Sternberg MM, et al: Comparative studies of impurities in intravenous immunoglobulin preparations. *Rev Infect Dis* 1986;8(suppl 4): S382-S390.

3. Mitra G, Wong MF, Mozen M, et al: Elimination of infectious retroviruses during preparation of immunoglobulins. *Transfusion* 1986;26:394-397.

See adjacent page for brief summary of prescribing information.



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Gamimune® N supplies a broad spectrum of opsonic and neutralizing IgG antibodies for the prevention or attenuation of a wide variety of infectious diseases. As Gamimune® N is administered intravenously, essentially 100% of the infused IgG antibodies are immediately available in the recipient's circulation. A relatively rapid fall in serum IgG level in the first week post-infusion is to be expected; this decrease averages 50% of the peak level achieved immediately post-infusion and is mainly due to the equilibration of IgG between the plasma and the extravascular space. The *in vivo* half-life of Gamimune® N equals or exceeds the three week half-life reported for IgG in the literature, but individual patient variation in half-life has been observed. Thus, this variable as well as the amount of immune globulin administered per dose is important in determining the frequency of administration of the drug for each individual patient.

The intravenous administration of solutions of maltose has been studied by several investigators. Healthy subjects tolerated the infusions well, and no adverse effects were observed at a rate of 0.25 g maltose/kg body weight per hour. In safety studies conducted by Cutter Biological, infusions of 10% maltose administered at 0.27-0.62 g maltose/kg per hour to normal subjects produced either mild side effects (e.g., headache) or no adverse reaction. Following intravenous administrations of maltose, maltose was detected in the peripheral blood; there was a dose-dependent excretion of maltose and glucose in the urine and a mild diuretic effect. These alterations were well tolerated without significant adverse effects. The highest recommended infusion rate, 0.08 mL/kg body weight per minute (see DOSAGE AND ADMINISTRATION), is equivalent to 0.48 g maltose/kg body weight per hour.

The buffer capacity of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N is 16.5 mEq/L (~0.3 mEq/g protein); a dose of 150-400 mg/kg (3-8 mL/kg) body weight therefore represents an acid load of 0.0495-0.1320 mEq/kg body weight. The total buffering capacity of whole blood in a normal individual is 45-50 mEq/L of blood, or 3.6 mEq/kg body weight. Thus, the acid load delivered in the largest dose of Gamimune® N would be neutralized by the buffering capacity of whole blood alone, even if the dose were infused instantaneously. (An infusion usually lasts several hours.)

In Phase I human studies, no change in arterial blood pH measurements was detected following the intravenous administration of Gamimune® N at a dose of 150 mg/kg body weight; following a dose of 400 mg/kg body weight in 37 patients, there were no clinically important differences in mean venous pH or bicarbonate measurements in patients who received Gamimune® N compared with those who received a chemically modified intravenous immunoglobulin preparation with a pH of 6.8.

In patients with limited or compromised acid-base compensatory mechanisms, consideration should be given to the effect of the additional acid load Gamimune® N might present.

INDICATIONS AND USAGE

Immunodeficiency Syndromes: Gamimune® N is indicated for the maintenance treatment of patients who are unable to produce sufficient amounts of IgG antibodies. Usage of Gamimune® N may be preferred to that of intramuscular immunoglobulin preparations, especially in patients who require an immediate increase in intravascular immunoglobulin levels, in patients with a small muscle mass, and in patients with bleeding tendencies in whom intramuscular injections are contraindicated. It may be used in disease states such as congenital agammaglobulinemia (e.g., X-linked agammaglobulinemia), common variable hypogammaglobulinemia, X-linked immunodeficiency with hyper IgM and in severe combined immunodeficiency.

Idiopathic Thrombocytopenic Purpura (ITP): Investigations in both children and adults have shown that Gamimune® N may initiate a therapeutic increase in the platelet count. In clinical studies of Gamimune® N, five of six (83.3%) children and 10 of 16 (62.5%) adults with acute or chronic ITP demonstrated clinically significant increments in the platelet count during or following an initial treatment course with Gamimune® N at a dose of 400 mg/kg body weight daily for five days. The duration of the platelet rise following treatment of ITP with Gamimune® N was variable, ranging from several days up to 12 months or more. Several ITP patients demonstrated continuing responsiveness over many months to intermittent Gamimune® N 400 mg/kg body weight single dose maintenance courses. Two of three children with acute ITP treated with Gamimune® N rapidly went into complete remission. However, childhood ITP may respond spontaneously without treatment. Four patients with refractory ITP were able to undergo major surgical procedures as a result of the rapid rise in platelet count associated with Gamimune® N treatment.

In addition, one patient with severe thrombocytopenia due to post-transfusion purpura (PLA¹ alloimmune antibody with platelet anti PLA¹ specificity) also responded to treatment with Gamimune® N, at a dose of 400 mg/kg body weight daily for 5 days; there was a rapid rise in the platelet count commencing on the third day of treatment.

It is presently not possible to predict which patients with ITP will respond to therapy, although the increase in platelet counts in children seems to be better than that of adults. In clinical situations in which a rapid rise in platelet count is needed to control bleeding or to allow a patient with ITP to undergo surgery, administration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N should be considered; in patients in whom a response is achieved, the rise of platelets is generally rapid (within 1-5 days), transient (most often lasting from several days to several weeks) and should not be considered curative. In some patients who relapse, a maintenance dose of Gamimune® N administered every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels (see DOSAGE AND ADMINISTRATION).

CONTRAINDICATIONS

Gamimune® N is contraindicated in individuals who are known to have had an anaphylactic or severe systemic response to Immune Globulin (Human). Individuals with selective IgA deficiencies who have known antibody against IgA (anti-IgA antibody) should not receive Gamimune® N since these patients may experience severe reactions to the IgA which may be present.

WARNINGS

Gamimune® N should be administered only intravenously as the intramuscular and subcutaneous routes have not been evaluated.

Gamimune® N may, on rare occasions, cause a precipitous fall in blood pressure and a clinical picture of anaphylaxis, even when the patient is not known to be sensitive to immune globulin preparations. These reactions may be related to the rate of infusion. Accordingly, the infusion rate given under DOSAGE AND ADMINISTRATION should be closely followed, at least until the physician has had sufficient experience with a given patient. The patient's vital signs should be monitored continuously and careful observation made for any symptoms throughout the entire infusion. Epinephrine should be available for the treatment of an acute anaphylactic reaction.

PRECAUTIONS

General: Any vial that has been entered should be used promptly. Partially used vials should be discarded. Do not use if turbid. Solution which has been frozen should not be used.

Drug Interactions: If dilution is required, Gamimune® N may be diluted with 5% dextrose in water (D5/W). No other drug interactions or compatibilities have been evaluated. It is recommended that infusion of Gamimune® N be given by a separate line, by itself, without mixing with other intravenous fluids or medications the patient might be receiving.

Pregnancy Category C: Animal reproduction studies have not been conducted with Gamimune® N. It is not known whether Gamimune® N can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Gamimune® N should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

In a study of 37 patients with immunodeficiency syndromes receiving Gamimune® N at a monthly dose of 400 mg/kg body weight, reactions were seen in 5.2% of the infusions of Gamimune® N. Symptoms reported with Gamimune® N included malaise, a feeling of faintness, fever, chills, headache, nausea, vomiting, chest tightness, dyspnea and chest, back or hip pain. In addition, mild erythema following infiltration of Immune Globulin Intravenous (Human) 5% (in 10% Maltose), pH 4.25—Gamimune® N at the infusion site was reported in some cases.

In further studies of Gamimune® N in the treatment of both adult and pediatric patients with ITP, systemic reactions were noted in only 4 of 154 (2.6%) infusions, and all but one occurred at rates of infusion greater than 0.04 mL/kg body weight per minute. The symptoms reported included chest tightness, a sense of tachycardia (pulse was 84 beats per minute), and a burning sensation in the head; these symptoms were all mild and transient. Erythema, pain, phlebitis, or eczematous reactions at the infusion site were also reported following infusion of Gamimune® N to adult ITP patients. These reactions occurred in 11.6% of the infusions of Gamimune® N and in 43.8% of the adult ITP population. No pediatric patients experienced localized reactions at the infusion site.

In the studies undertaken to date, other types of reactions have not been reported with Gamimune® N. It may be, however, that adverse effects will be similar to those previously reported with intravenous and intramuscular immunoglobulin administration. Potential reactions, therefore, may also include anxiety, flushing, wheezing, abdominal cramps, myalgias, arthralgias, and dizziness; rash has been reported only rarely. Reactions to intravenous immunoglobulin tend to be related to the rate of infusion.

True anaphylactic reactions to Gamimune® N may occur in recipients with documented prior histories of severe allergic reactions to intramuscular immunoglobulin, but some patients may tolerate cautiously administered intravenous immunoglobulin without adverse effects. Very rarely an anaphylactoid reaction may occur in patients with no prior history of severe allergic reactions to either intramuscular or intravenous immunoglobulin.

DOSAGE AND ADMINISTRATION

Immunodeficiency Syndromes: The usual dosage of Gamimune® N for prophylaxis in immunodeficiency syndromes is 100-200 mg/kg (2-4 mL/kg) of body weight administered approximately once a month by intravenous infusion. If the clinical response is inadequate, or the level of IgG achieved in the circulation is felt to be insufficient, the dosage may be given more frequently or increased as high as 400 mg/kg (8 mL/kg) body weight.

Idiopathic Thrombocytopenic Purpura: An increase in platelet count has been observed in children and some adults with acute or chronic ITP receiving Gamimune® N, 400 mg/kg body weight daily for five days. A response usually occurs within several days and is maintained for a variable period of time. In general a response is seen less often in adults than in children. In patients who have shown a response, a maintenance dose of Gamimune® N of 400 mg/kg body weight administered as a single dose every several weeks may be of benefit once the platelet count decreases to clinically hazardous levels.

Investigations indicate that Gamimune® N is well-tolerated and less likely to produce side effects when infused at the indicated rate. It is recommended that Gamimune® N be infused by itself at a rate of 0.01 to 0.02 mL/kg body weight per minute for 30 minutes; if well tolerated, the rate may be gradually increased to a maximum of 0.08 mL/kg body weight per minute. If side effects occur, the rate may be reduced, or the infusion interrupted until symptoms subside. The infusion may then be resumed at the rate which is comfortable for the patient.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

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14-7640-001

Mar 1986

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Drug of Choice in PCP

Bactrim I.V. is the drug of choice for treatment of *Pneumocystis carinii* pneumonia (PCP).¹ Please note, though, that AIDS patients may not tolerate or respond to treatment in the same way as non-AIDS patients.

The incidence of side effects—particularly rash, fever and leukopenia—is greatly increased in AIDS patients. Discontinue therapy at the first sign of skin rash or any adverse reaction.

High survival rate after first episodes of PCP in AIDS patients

In a multicenter evaluation of 185 AIDS patients who received trimethoprim plus sulfamethoxazole for their first episodes of PCP, 58% completed therapy with this combination alone. Among these patients, the rate of immediate survival was 82%.²

Immediate survival
with
TMP/SMX alone



Adapted from report of a National Heart, Lung and Blood Institute Workshop.²

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

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References: 1. *Medical Letter* 28(706):9-16, Jan 31, 1986. 2. Murray JF, et al: *N Engl J Med* 310(25):1682-1688, Jun 21, 1984.

Drug of choice in PCP¹

Bactrim™ I.V. Infusion

(16 mg trimethoprim and 80 mg sulfamethoxazole per mL)

Before prescribing, please consult complete product information, a summary of which follows:

CONTRAINDICATIONS: Hypersensitivity to trimethoprim or sulfonamides; documented megaloblastic anemia due to folate deficiency; pregnancy at term and during the nursing period; infants less than two months of age.

WARNINGS: FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS.

BACTRIM SHOULD BE DISCONTINUED AT THE FIRST APPEARANCE OF SKIN RASH OR ANY SIGN OF ADVERSE REACTION. Clinical signs, such as rash, sore throat, fever, arthralgia, cough, shortness of breath, pallor, purpura or jaundice, may be early indications of serious reactions. In rare instances a skin rash may be followed by more severe reactions, such as Stevens-Johnson syndrome, toxic epidermal necrolysis, hepatic necrosis or serious blood disorder. Perform complete blood counts frequently.

BACTRIM SHOULD NOT BE USED IN THE TREATMENT OF STREPTOCOCCAL PHARYNGITIS. Clinical studies have documented that patients with group A β -hemolytic streptococcal tonsillopharyngitis have a greater incidence of bacteriologic failure when treated with Bactrim than with penicillin.

Bactrim I.V. Infusion contains sodium metabisulfite, which may cause allergic-type reactions in susceptible persons, especially asthmatics or atopic nonasthmatic persons.

PRECAUTIONS:

General: Give with caution to patients with impaired renal or hepatic function, possible folate deficiency (e.g., elderly, chronic alcoholics, patients on anticonvulsants, with malabsorption syndrome, or in malnutrition states) and severe allergies or bronchial asthma. In glucose-6-phosphate dehydrogenase deficient individuals, hemolysis may occur, frequently dose-related.

Local irritation and inflammation due to extravascular infiltration of the infusion have been observed. If these occur, discontinue infusion and restart at another site.

Use in the Elderly: May be increased risk of severe adverse reactions in elderly, particularly with complicating conditions, e.g., impaired kidney and/or liver function, concomitant use of other drugs. Severe skin reactions, generalized bone marrow suppression (see WARNINGS and ADVERSE REACTIONS) or a specific decrease in platelets (with or without purpura) are most frequently reported severe adverse reactions in elderly. In those concurrently receiving certain diuretics, primarily thiazides, increased incidence of thrombocytopenia with purpura reported. Make appropriate dosage adjustments for patients with impaired kidney function (see DOSAGE AND ADMINISTRATION).

Use in the Treatment of *Pneumocystis Carinii* Pneumonitis in Patients with Acquired Immunodeficiency Syndrome (AIDS): Because of unique immune dysfunction, AIDS patients may not tolerate or respond to Bactrim in same manner as non-AIDS patients. Incidence of side effects, particularly rash, fever, leukopenia, with Bactrim in AIDS patients treated for *Pneumocystis carinii* pneumonitis reported to be greatly increased compared with incidence normally associated with Bactrim in non-AIDS patients.

Laboratory Tests: Perform appropriate culture and susceptibility studies before and throughout treatment. Complete blood counts should be done frequently; if a significant reduction in the count of any formed blood element is noted, discontinue Bactrim. Perform urinalyses with careful microscopic examination and renal function tests during therapy, particularly for patients with impaired renal function.

Drug Interactions: In elderly patients concurrently receiving certain diuretics, primarily thiazides, an increased incidence of thrombocytopenia with purpura has been reported. Bactrim may prolong the prothrombin time in patients who are receiving the anticoagulant warfarin. Keep this in mind when Bactrim is given to patients already on anticoagulant therapy and reassess coagulation time. Bactrim may inhibit the hepatic metabolism of phenytoin. Given at a common clinical dosage, it increased the phenytoin half-life by 39% and decreased the phenytoin metabolic clearance rate by 27%. When giving these drugs concurrently, be alert for possible excessive phenytoin effect. Sulfonamides can displace methotrexate from plasma protein binding sites, thus increasing free methotrexate concentrations.

Drug/Laboratory Test Interactions: Bactrim, specifically the trimethoprim component, can interfere with a serum methotrexate assay as determined by the competitive binding protein technique (CBPA) when a bacterial dihydrofolate reductase is used as the binding protein. No interference occurs if methotrexate is measured by a radioimmunoassay (RIA). The presence of trimethoprim and sulfamethoxazole may also interfere with the Jaffe alkaline picrate reaction assay for creatinine, resulting in overestimations of about 10% in the range of normal values.

Carcinogenesis, Mutagenesis, Impairment of Fertility: **Carcinogenesis:** Long-term studies in animals to evaluate carcinogenic potential not conducted with Bactrim I.V. Infusion. **Mutagenesis:** Bacterial mutagenic studies not performed with sulfamethoxazole and trimethoprim in combination. Trimethoprim demonstrated to be nonmutagenic in the Ames

BACTRIM™ I.V. Infusion (trimethoprim and sulfamethoxazole/Roche)

assay. No chromosomal damage observed in human leukocytes cultured *in vitro* with sulfamethoxazole and trimethoprim alone or in combination; concentrations used exceeded blood levels of these compounds following therapy with Bactrim. Observations of leukocytes obtained from patients treated with Bactrim revealed no chromosomal abnormalities. **Impairment of Fertility:** Bactrim I.V. Infusion not studied in animals for evidence of impairment of fertility. Studies in rats at oral dosages as high as 70 mg/kg trimethoprim plus 350 mg/kg sulfamethoxazole daily showed no adverse effects on fertility or general reproductive performance.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Trimethoprim and sulfamethoxazole may interfere with folic acid metabolism; use during pregnancy only if potential benefit justifies potential risk to fetus. Nonteratogenic Effects: See CONTRAINDICATIONS section.

Nursing Mothers: See CONTRAINDICATIONS section.

Pediatric Use: Not recommended for infants under two months (see CONTRAINDICATIONS section).

ADVERSE REACTIONS: Most common are gastrointestinal disturbances (nausea, vomiting, anorexia) and allergic skin reactions (such as rash and urticaria). **FATALITIES ASSOCIATED WITH THE ADMINISTRATION OF SULFONAMIDES, ALTHOUGH RARE, HAVE OCCURRED DUE TO SEVERE REACTIONS, INCLUDING STEVENS-JOHNSON SYNDROME, TOXIC EPIDERMAL NECROLYSIS, FULMINANT HEPATIC NECROSIS, AGRANULOCYTOSIS, APLASTIC ANEMIA AND OTHER BLOOD DYSCRASIAS (SEE WARNINGS SECTION).** Local reaction, pain and slight irritation on I.V. administration infrequent. Thrombophlebitis rarely observed.

Hematologic: Agranulocytosis, aplastic anemia, thrombocytopenia, leukopenia, neutropenia, hemolytic anemia, megaloblastic anemia, hypoprothrombinemia, methemoglobinemia, eosinophilia. **Allergic Reactions:** Stevens-Johnson syndrome, toxic epidermal necrolysis, anaphylaxis, allergic myocarditis, erythema multiforme, exfoliative dermatitis, angioedema, drug fever, chills, Henoch-Schoenlein purpura, serum sickness-like syndrome, generalized allergic reactions, generalized skin eruptions, conjunctival and scleral injection, photosensitivity, pruritus, urticaria and rash. Periarthritis nodosa and systemic lupus erythematosus have been reported. **Gastrointestinal:** Hepatitis (including cholestatic jaundice and hepatic necrosis), elevation of serum transaminase and bilirubin, pseudomembranous enterocolitis, pancreatitis, stomatitis, glossitis, nausea, emesis, abdominal pain, diarrhea, anorexia. **Genitourinary:** Renal failure, interstitial nephritis, BUN and serum creatinine elevation, toxic nephrosis with oliguria and anuria, crystalluria. **Neurologic:** Aseptic meningitis, convulsions, peripheral neuritis, ataxia, vertigo, tinnitus, headache. **Psychiatric:** Hallucinations, depression, apathy, nervousness. **Endocrine:** Sulfonamides bear certain chemical similarities to some goitrogens, diuretics (acetazolamide and the thiazides) and oral hypoglycemic agents; cross-sensitivity may exist. Diuresis and hypoglycemia have occurred rarely in patients receiving sulfonamides. **Musculoskeletal:** Arthralgia, myalgia. **Respiratory:** Pulmonary infiltrates. **Miscellaneous:** Weakness, fatigue, insomnia.

DOSAGE AND ADMINISTRATION: CONTRAINDICATED IN INFANTS LESS THAN TWO MONTHS OF AGE. CAUTION—BACTRIM I.V. INFUSION MUST BE DILUTED IN 5% DEXTROSE IN WATER SOLUTION PRIOR TO ADMINISTRATION. DO NOT MIX WITH OTHER DRUGS OR SOLUTIONS. RAPID INFUSION OR BOLUS INJECTION MUST BE AVOIDED. *(Children and Adults):*

PNEUMOCYSTIS CARINII PNEUMONITIS: Total daily dose is 15 to 20 mg/kg (based on the trimethoprim component) given in three or four equally divided doses every 6 to 8 hours for up to 14 days. One investigator noted that a total daily dose of 10 to 15 mg/kg was sufficient in 10 adults with normal renal function.

SEVERE URINARY TRACT INFECTIONS AND SHIGELLOSIS: Total daily dose is 8 to 10 mg/kg (based on the trimethoprim component) given in two to four equally divided doses every 6, 8 or 12 hours for up to 14 days for severe urinary tract infections and 5 days for shigellosis. Maximum recommended daily dose is 60 ml. **Renal Impaired:** Creatinine clearance above 30 ml/min, give usual dosage; 15-30 ml/min, give one-half the usual regimen; below 15 ml/min, use not recommended.

Method of Preparation: Bactrim I.V. Infusion must be diluted. EACH 5 ML SHOULD BE ADDED TO 125 ML OF 5% DEXTROSE IN WATER. After diluting do not refrigerate; use within 6 hours. If a dilution of 5 ml/100 ml of 5% dextrose in water is desired, use within 4 hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one.

Multidose Vials: After initial entry into the vial, use remaining contents within 48 hours. The following infusion systems are satisfactory: unit-dose glass containers; unit-dose polyvinyl chloride and polyolefin containers.

Dilution: ADD EACH 5 ML OF BACTRIM I.V. INFUSION TO 125 ML OF 5% DEXTROSE IN WATER.

NOTE: When fluid restriction is desirable, add each 5 ml to 75 ml of 5% dextrose in water; mix just prior to use and administer within two hours. If there is cloudiness or evidence of crystallization, discard solution and prepare a fresh one. DO NOT MIX BACTRIM I.V. INFUSION—5% DEXTROSE IN WATER WITH DRUGS OR SOLUTIONS IN THE SAME CONTAINER.

ADMINISTRATION: Administer by I.V. infusion over 60 to 90 minutes. Avoid rapid infusion or bolus injection. Do not give intramuscularly.

HOW SUPPLIED: 5-ml ampuls, 5-ml vials and 10-ml vials—boxes of 10; 30-ml and 50-ml multidose vials—boxes of 1. Each 5 ml contains 80 mg trimethoprim (16 mg/ml) and 400 mg sulfamethoxazole (80 mg/ml) for infusion with 5% dextrose in water.

STORE AT ROOM TEMPERATURE (15°-30°C or 59°-86°F). DO NOT REFRIGERATE.

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Brief Summary of Prescribing Information (1) 10-84 For complete prescribing information please consult product literature

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents.

Squamous Cell Carcinoma — Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas — Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma — Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1% the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary — This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of the lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrous exudation into alveoli, producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonia.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain F_iO₂ at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions — In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes — These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

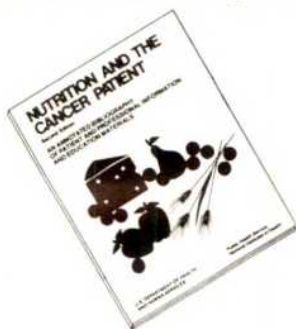
Other — Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban®, or a combination of any or all of these.

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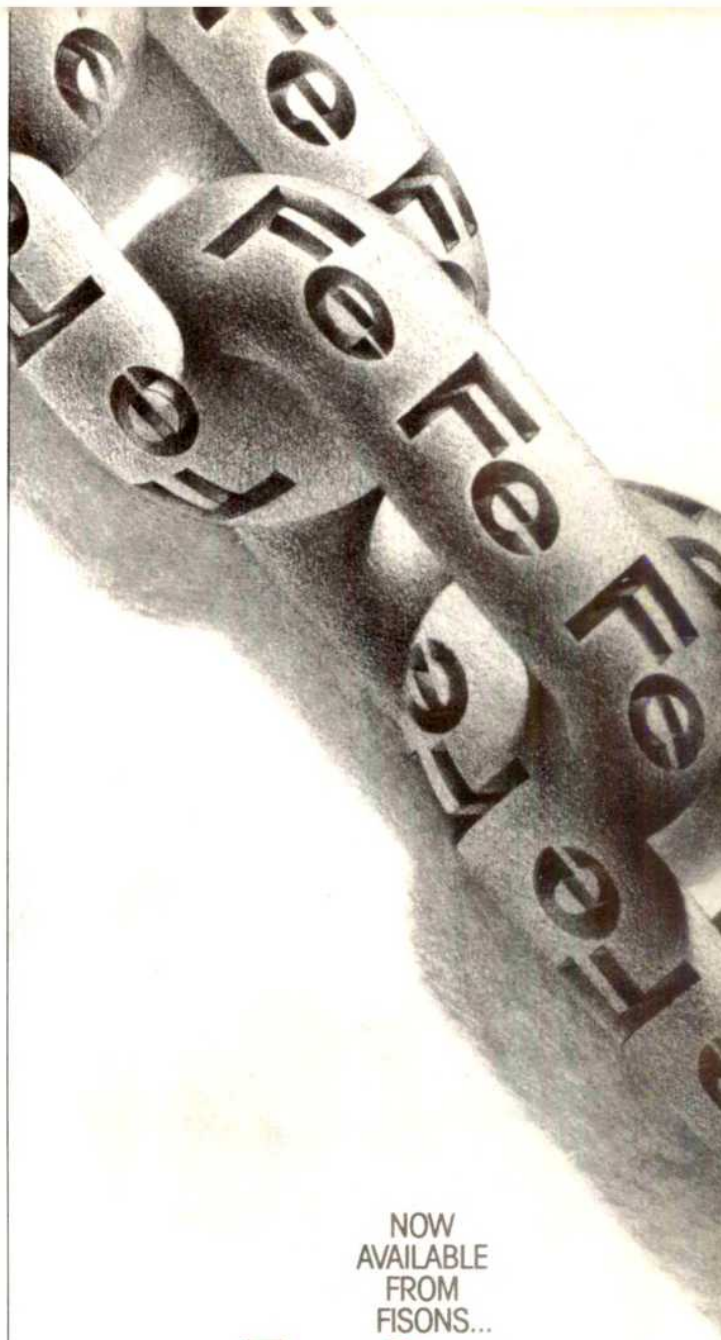
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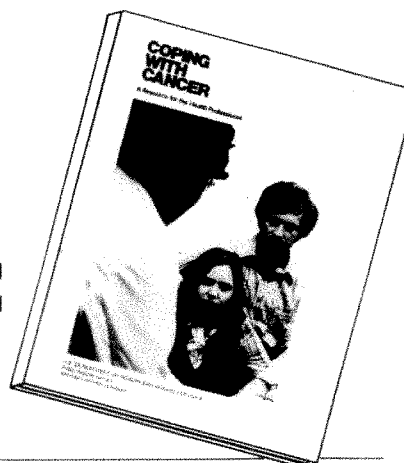
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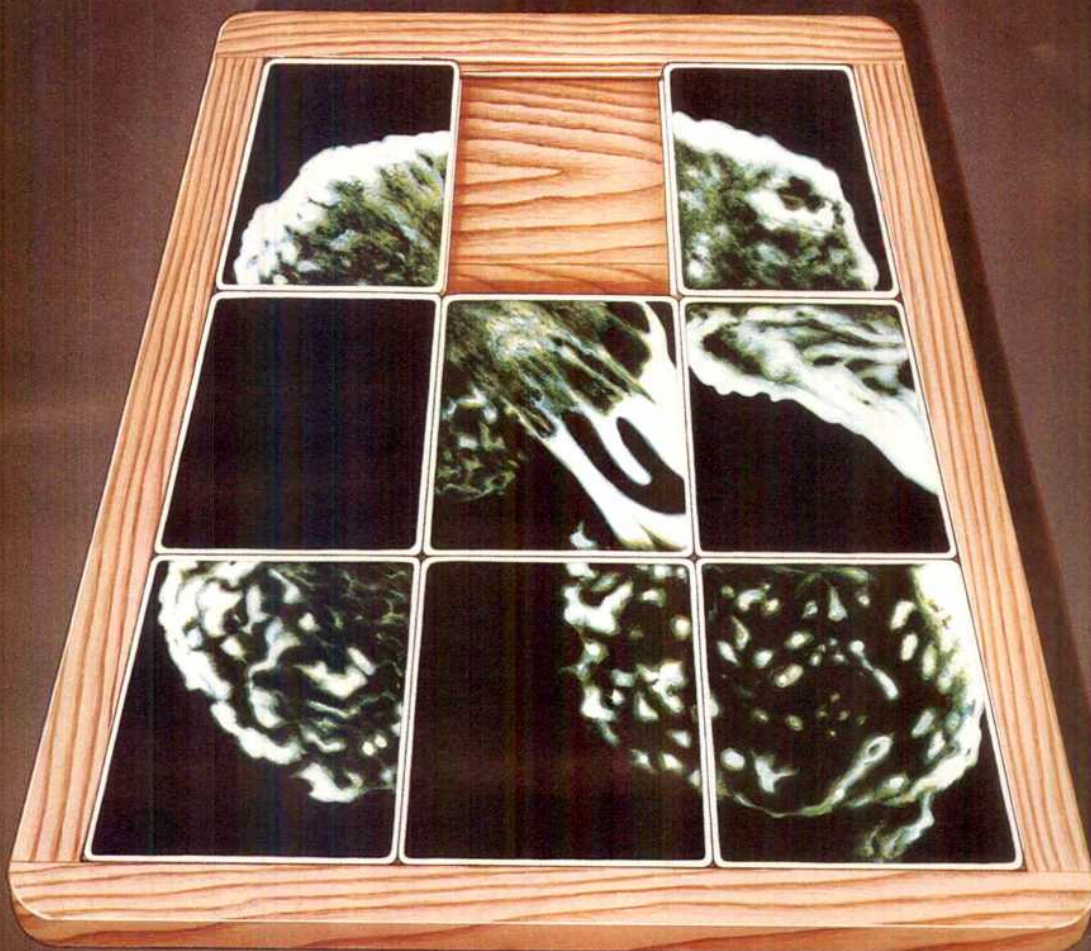
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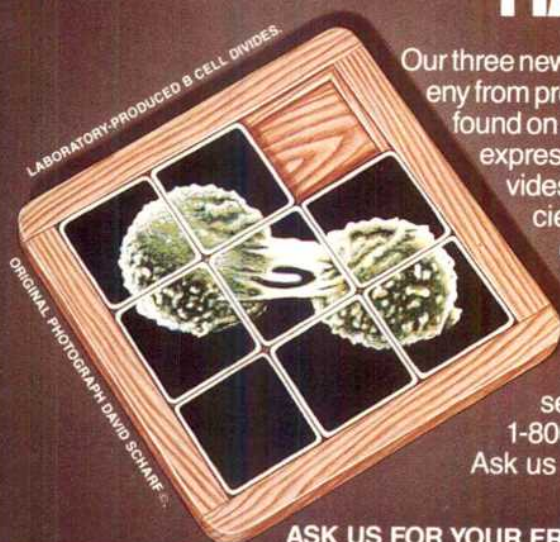


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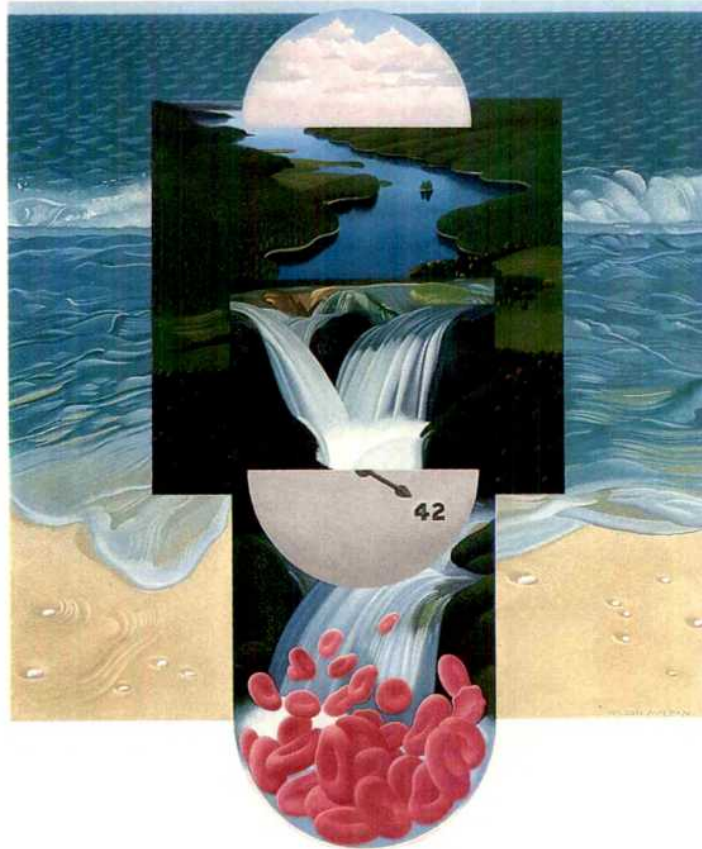
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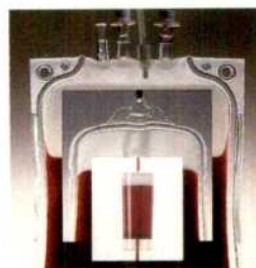


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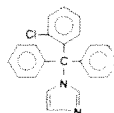
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The troche dosage form is a large, slowly-dissolving tablet (lozenge) containing 10 mg of clotrimazole dispersed in dextrose, microcrystalline cellulose, povidone, and magnesium stearate.

Clinical Pharmacology

Clotrimazole is a broad-spectrum antifungal agent that inhibits the growth of pathogenic yeasts by altering the permeability of cell membranes. It exhibits fungicidal activity *in vitro* against *Candida albicans* and other species of the genus *Candida*. No single-step or multiple-step resistance to clotrimazole has developed during successive passages of *Candida albicans* in the laboratory.

After oral administration of a 10 mg clotrimazole troche to healthy volunteers concentrations sufficient to inhibit most species of *Candida* persist in saliva for up to three hours following the approximately 30 minutes needed for a troche to dissolve. The long-term effective concentration of drug in saliva appears to be related to the slow release of clotrimazole from the oral mucosa to which the drug is apparently bound. Repetitive dosing at three hour intervals maintains effective salivary levels above the minimum inhibitory concentration of most strains of *Candida*. In another study the mean serum concentrations were 4.98 $\pm$ 3.7 and 3.23 $\pm$ 1.4 nanograms/ml at 30 and 60 minutes respectively.

Indications and Usage

Mycelex[®] Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications

Mycelex[®] Troches are contraindicated in patients who are hypersensitive to any of its components.

Warning

Mycelex[®] Troches are not indicated for the treatment of systemic mycoses.

Precautions

Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with pre-existing hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18 month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use

Safety and effectiveness of clotrimazole in children below the age of 3 years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions

Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in twenty patients.

Overdosage

No data available.

Drug Abuse and Dependence

No data available.

Dosage and Administration

Mycelex[®] Troches are administered only as a lozenge that must be slowly dissolved in the mouth. The recommended dose is one troche five times a day for fourteen consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied

Mycelex[®] Troches, white discoid, uncoated tablets are supplied in bottles of 70 and 140. Mycelex Troches are also available for institutional use in foil packages of 70 tablets. Each tablet will be identified with the following: Miles 095.

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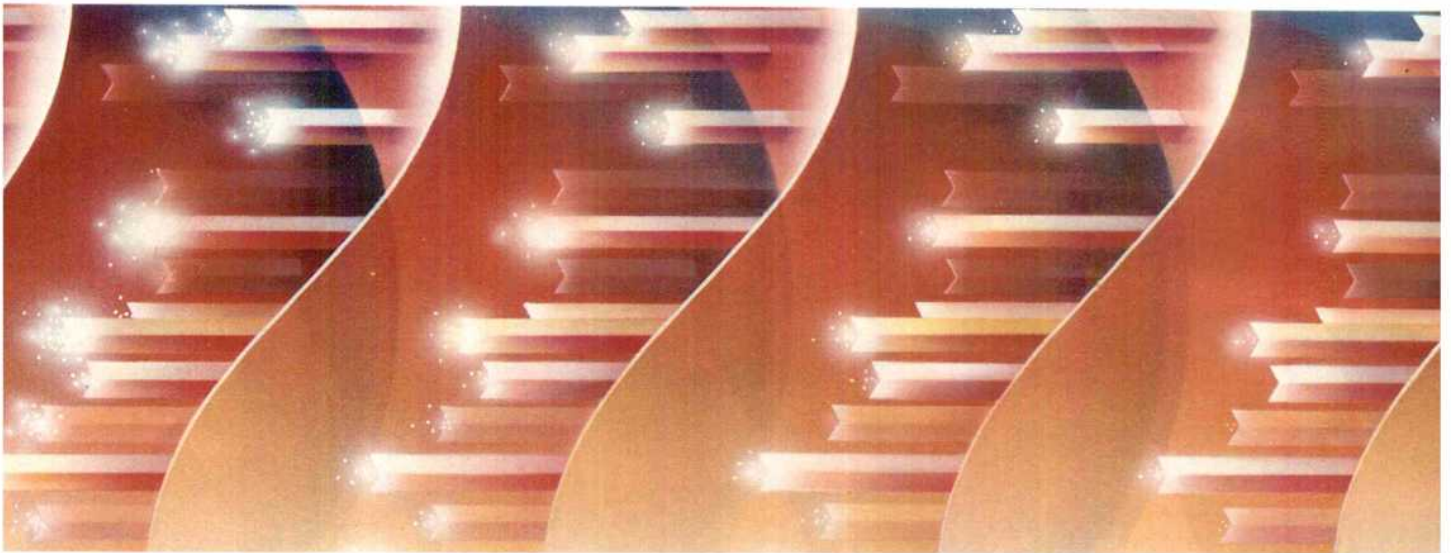
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REVIEW

Structure-Function Relationship of Human Von Willebrand Factor

By Jean-Pierre Girma, Dominique Meyer, Cornelis L. Verweij, Hans Pannekoek, and Jan J. Sixma

THE GLYCOPROTEIN von Willebrand factor (vWF) is synthesized by endothelial cells and megakaryocytes and is absent or abnormal in von Willebrand's disease (vWD).¹ It is multimeric (0.5 or 1 to at least 20×10^6 daltons² and its concentration in plasma is approximately 7 to 10 $\mu\text{g/mL}$. vWF serves as carrier protein for coagulation factor VIII (absent in hemophilia A) and functions as an adhesive protein for platelets. This latter function is observed at relatively high shear rates, prevailing in arteries and the microcirculation.^{3,4} vWF from plasma appears to bind to the injured vessel wall, after which platelets may interact with it.⁵ Whether vWF supports the initial contact of platelets with the vessel wall or the subsequent spreading has been disputed.^{6,7} Recent data indicate that it may indeed support both⁸ and also has a role in thrombus formation.⁹ The normal vessel wall already contains some vWF,¹⁰ deposited there by the covering endothelial cells, and this vWF is able to support about one-half of the platelet adhesion. Plasma vWF is needed, however, to obtain optimal adhesion.^{11,12} Blood platelets contain vWF in their α -granules. This pool of vWF also appears to be involved in platelet adhesion.¹³

INTERACTION OF vWF WITH PLATELETS

vWF interacts with platelets through two membrane binding sites. Glycoprotein Ib(GpIb) is primarily involved in platelet-vessel wall adhesion. The glycoprotein IIb-IIIa (GpIIb-IIIa) complex plays a secondary role in adhesion,¹⁴⁻¹⁶ and is mainly involved in platelet-platelet interaction. The latter is also defective in vWD.⁹ In *in vitro* studies the role of GpIb in the interaction of vWF with platelets is most often studied in the presence of the obsolete antibiotic ristocetin which causes binding of vWF to GpIb on the platelet membrane and subsequently platelet aggregation. The role of GpIb in this process was first demonstrated by the absent binding of vWF to platelets in the Bernard-Soulier syndrome, characterized by large platelets lacking GpIb.¹⁷ This role was further confirmed in studies with monoclonal antibodies (MAbs) against GpIb.^{18,19} More recently, it was demonstrated that platelets also bind vWF when stimulated with thrombin or ADP.^{20,21} This thrombin-induced vWF binding was defective in Glanzmann's thrombasthenia,²² which is characterized by absent platelet aggregation and lack or abnormality of GpIIb-IIIa.²³ The role of GpIIb-IIIa in the thrombin-induced binding of vWF was subsequently

also confirmed in studies with MAbs to GpIIb-IIIa²⁴ and to vWF.¹⁴

INTERACTION WITH THE VESSEL WALL

The binding site for vWF in the vessel wall is at present still unknown. vWF binds to monomeric and fibrillar collagen type III and to fibrillar collagen type I,^{25,26} both of which are present in the vessel wall. Platelet adhesion to monomeric and fibrillar collagen type I and III at high shear rates is also dependent on vWF.²⁷ Recent data have, however, shed doubt on the notion that collagen represents the only binding site for vWF in the subendothelium of blood vessels.²⁸ A MAb against vWF inhibiting binding of vWF to collagen had no influence on the binding of vWF to the matrix of cultured endothelial and smooth muscle cells, whereas another MAb that inhibited binding of vWF to the matrix of these cells had no effect on the binding of vWF to collagen.²⁹ The latter MAb also inhibited the adhesion of platelets to these matrices. Such data indicate that there is an as yet unidentified substance in the matrix of these cells to which vWF binds. The binding of vWF to collagen may be of importance, however, in a hemostatic situation, in which fibrillar collagen becomes exposed to flowing blood at some distance from the vessel lumen.

THE ESSENTIAL INFORMATION FOR THE FUNCTION OF vWF RESIDES IN THE MONOMER

In the past, studies on the structure-function relationship of vWF have been difficult. The difficulty was related to the complex nature of vWF and to the observations that its multimeric structure was required for optimal function, which implied that conformational aspects of the molecule were of major importance for its function.^{30,31} In recent years, new data have been acquired indicating that the essential information regarding the function of vWF resides in its

From INSERM U.143, Hôpital Bicêtre, Paris; the Department of Molecular Biology, Central Laboratory of the Blood Transfusion Service, Amsterdam; and the Department of Hematology, University Hospital, Utrecht, The Netherlands.

Submitted January 8, 1987; accepted April 29, 1987.

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basic subunit. The importance of the multimeric nature of vWF for its function is now primarily ascribed to its ability to provide a high local density of binding sites by presenting many repeating subunits. The observations leading to this revision in insight were the following: vWF binding to platelets showed higher affinity for larger multimers, but multimers with a lower mol wt and even subunits obtained after reduction also bound.^{30,32} The support of platelet adhesion was also to an unexpected extent independent of the multimeric size of vWF.³³ This was most convincingly demonstrated in perfusion studies in which platelet adhesion to collagen was supported by a dimeric proteolytic fragment of vWF.³⁴ Moreover, specific functions of vWF appeared to be inhibited when a single epitope was occupied by MAbs to vWF.^{6,14,29,35,36} Thus, the essential information for the functions of vWF resides in its single polypeptide chain and is not brought about by the conformational changes caused by the polymerization into large multimers.

STRUCTURE OF vWF

Much new information obtained recently about the structure of vWF has been helpful in interpretation of the data on localization of functional domains in various parts of the molecule. In particular, data obtained by molecular cloning of cDNA of vWF³⁷⁻⁴³ together with amino acid sequencing of vWF² provide a firm framework of reference. The full length of the vWF-cDNA is ~8,900 base pairs, and it has a continuous open reading frame encoding 2,813 amino acids (Fig 1). This implies a translation product of 309 kd, much larger than the 225 kd or 2,050 amino acids of vWF. This discrepancy is explained by the observation that the nucleotide sequence coding for mature vWF starts only at base 2,290. This implies that the vWF precursor protein is 763 amino acid residues larger than the mature protein (Fig 1). The first 22 amino acids are a typical signal peptide. The subsequent 741 amino acids, yielding a calculated mol wt of 81 kd, correspond to a protein that has been called von Willebrand antigen II (vWAgII).^{38,44} This protein is split off from vWF in the endothelial cell. Together with vWF, it is stored in Weibel-Palade bodies.⁴⁵ It is also present in mega-

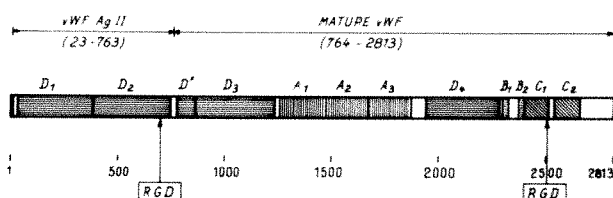


Fig 1. Schematic representation of vWF precursor showing (dark area) the signal peptide (residues 1 through 22), the position of vWAgII (residues 23 through 763), and the position of the mature vWF (residues 764 through 2,813). N-terminus of the molecule is at left. Hatched areas indicate internal homologous regions in the sequence of the precursor as recently described by Verweij et al,⁴³ with the triplicated A domains, the duplicated B and C domains, the quadruplicated D domain, and the partially homologous domain D'. Arrows indicate the position of RGD sequences already identified in the GpIb-IIIa binding domain of fibrinogen and fibronectin.

karyocytes and platelets, where it is stored in α -granules.⁴⁶ vWAgII is also absent in some cases of vWD.⁴⁷ Its functional role is as yet unknown, but it is interesting that it contains an Arg-Gly-Asp (RGD) sequence found in adhesive proteins binding to GpIb-IIIa.⁴³

vWF together with its precursor protein has a number of striking internal homologies^{41,43} summarized in Fig 1: the D domain is 350 to 400 amino acid residues long, and this is present four times in the molecule. Part of this domain (96 amino acids long) is present at the amino-terminus of the mature vWF protein (indicated as D' in Fig 1). The A domain is present three times between residues 1,242 and 1,875 of the precursor. The much shorter B and C domains are each duplicated. They are near the carboxy-terminus of the molecule.

Mature vWF itself consists of a single chain of 2,050 amino acids with an amino-terminal serine and a carboxy-terminal lysine (Fig 2). Twenty-two probable glycosylation sites have been located on the subunit.² Twelve are N-glycosylation sites on asparagine, mainly consisting of a monosialylated biantennary glycan of the N-acetylgalactosaminic type.⁴⁸ Ten O-glycosylation sites are on threonine. Distribution of the carbohydrate side chains along the sequence of vWF is not uniform and most of the glycosylated sites are clustered on the N-terminal or the C-terminal portions of the molecule, with a large gap in the central part between residues 916 and 1,460 of mature vWF.² In addition, eight of the O-glycosylation sites also appear to be located in the narrow domains between residues 300 through 485 and

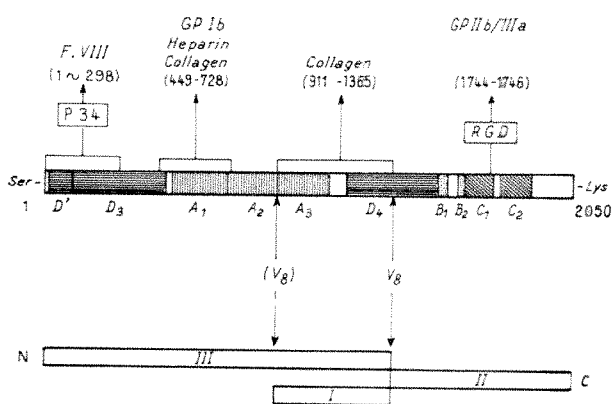


Fig 2. Schematic representation of mature vWF subunit. This scheme compares the position of the homologous regions described in Fig 1 with that of the domains responsible for binding of vWF to GpIb, GpIIb-IIIa, heparin, and collagen. The sequence extending from residues 449 through 728 contains a GpIb-⁶⁶ heparin,⁷⁸ and collagen⁷² binding domain. A second binding domain to collagen was identified between amino acids 911 through 1,365.⁷¹ The GpIIb-IIIa binding domain was identified on the carboxy-terminal end of vWF extending from residues 1,366 to 2,050.⁶⁶ The domain may comprise the RGD sequence located at residues 1,744 through 1,746 of mature vWF and identified in the GpIIb-IIIa binding site of fibrinogen and fibronectin. The position of hydrolysis of vWF by *S aureus* V-8 protease that allows three functional domains to separate (bottom): fragment Sp III: amino-terminal, and fragment Sp I, the central part of vWF subunit.

705 through 724. Due to glycosylation (18.7% carboxyhydrate) the actual apparent mol wt of the single chain is 270 to 280 kd.² In concentrates of vWF, smaller chains with mol wt between 190 and 120 kd have also been found.^{49,50} They represent proteolytic degradation products of vWF. The single chain of vWF is linked to another chain by firm disulphide bonds at the carboxy-terminal end of the molecule. The dimers that are thus formed are further linked to one another to form multimers by much weaker disulphide bonds at the amino-terminal end. An amino-terminal 34-kd fragment of the molecule is evidently not involved in the disulphide bonding, as it is easily split off by proteolytic enzymes such as plasmin and trypsin without any effect on the multimeric patterns.^{51,52} Whether it is the dimer^{33,53-55} or the tetramer^{56,57} that forms the protomer for the multimeric series of vWF present in plasma is still disputed.

Electron microscopical studies of vWF have shown that it consists of a linear arrangement of similar subunits. A large globular area was found at the amino-terminus with a rodlike portion at the center and a small globule at the carboxy-terminal end.⁵⁸⁻⁶¹

FUNCTIONAL DOMAINS ON vWF: GPIb-BINDING DOMAIN

The first functional domain to be localized on vWF was that interacting with GPIb. Trypsin digestion of vWF provided a 116-kd fragment of vWF that had some remaining ristocetin cofactor activity.⁶² A MAb inhibiting the interaction of vWF with platelets in the presence of ristocetin also recognized this fragment.⁵² Binding studies showed that this fragment associated with platelets in the presence of ristocetin.⁶³ After reduction, a 53- to 55-kd doublet was recognized by the specific MAb.⁵² Studies with digestion of vWF by staphylococcal protease V8 provided two main fragments of vWF: SpIII and SpII.⁶⁰ SpIII was shown to be a dimer of the amino-terminal 170-kd part of vWF and to contain the GPIb and collagen binding domains of vWF.^{64,65} This was demonstrated by using specific MAbs inhibiting binding of vWF to platelets and to collagen, by direct competitive binding studies between SpIII and vWF and by direct binding of purified labeled SpIII. Further digestion studies with subtilisin produced small fragments of SpIII. Fragments of 33 and 28 kd possessed the epitope for the MAb inhibiting binding of vWF to platelets in the presence of ristocetin.⁶⁵ More recently, a 48/52-kd doublet found after tryptic digestion and reduction was shown to interact with GPIb, as it retained the ability to inhibit platelet aggregation induced by vWF in the presence of ristocetin or by asialo-vWF in the absence of inducer.⁶⁶ In addition, it was established that the 48/52-kd species could be converted into a single 46-kd fragment after enzymatic removal of the carbohydrate side chains and that this 46-kd fragment as well as the parent 48/52-kd doublet also retained the ability to inhibit vWF binding to platelet GPIb. The 46-kd fragment is located between residues 449 and 728.⁶⁶

FUNCTIONAL DOMAINS: GPIIb-IIIa BINDING DOMAIN

A perhaps even more precise localization of the domain on vWF interacting with GPIIb-IIIa on platelets could be made

tentatively. Binding studies of the dimeric fragments of vWF obtained after digestion with *Staphylococcus aureus* V8 protease showed that the carboxy-terminal SpII fragment bound to thrombin-treated platelets.⁶⁵ Further cleavage of SpII with thermolysin produced a dimeric fragment with a mol wt of 86 kd possessing the epitope for the MAb that specifically inhibited the binding of vWF to thrombin-stimulated platelets.⁶⁵ Proteolytic digestion of vWF with trypsin also produced a series of fragments, of which the smallest was 80 kd, reacting with the same MAb.⁶⁷ This fragment also reacted with a MAb that was used in molecular cloning that appeared to recognize the carboxy-terminal end of vWF.⁴² These data suggest that the actual binding site cannot be localized at >40 kd from the carboxy-terminus. The binding of vWF to GPIIb-IIIa is specifically inhibited by oligopeptides containing the sequence Arg-Gly-Asp (-Ser) [RGD(S)].^{68,69} This sequence was first identified as the cell attachment site in fibronectin and subsequently recognized in many other adhesive proteins.⁷⁰ Peptides containing this sequence also inhibited the interaction of fibronectin and fibrinogen with GPIIb-IIIa complex on the platelet.^{68,69} The same sequence was also found in vWF at residues 1,744 through 1,746, ~300 amino acids from the carboxy-terminal end.^{2,40,43} It is plausible that this sequence is part of the binding site on vWF for GPIIb-IIIa.

FUNCTIONAL DOMAINS: COLLAGEN BINDING DOMAIN

The domain on vWF responsible for its binding to collagen has also received much attention. By means of *S. aureus* protease V8, the domain was localized on the amino-terminal SpIII fragment.⁶⁵ Similar studies with trypsin digestion initially localized the collagen binding domain on a 240-kd fragment obtained from the carboxy-terminal side of the molecule.⁵² A 48-kd fragment was then split off by further digestion. This fragment still bound to collagen and contained the epitope for the MAb inhibiting the binding of vWF to collagen.^{52,63} A fragment of similar size was also identified by Western blotting of a subtilisin digest of SpIII. After prolonged digestion and immunoadsorption, two fragments of 23 and 26 kd were found shifting to a slightly higher mol wt on reduction.⁶⁵ From the information obtained with *S. aureus* protease V8 and with trypsin digestion, a localization of the collagen binding domain situated between the GPIb binding domain and the GPIIb-IIIa binding domain could be deduced.

Better localization could be made on the basis of Western blotting of the *S. aureus* V8 protease digest of vWF. Apart from the main dimeric fragments SpII and SpIII produced by a primary cleavage (Glu 1,365-Glu 1,366), a third fragment SpI is present in much smaller amount and is released from the carboxy-terminal end of SpIII by a secondary cleavage (Glu 910-Gly 911).⁶⁴ SpI reacts in blotting experiments with MAbs inhibiting binding of vWF to collagen.⁷¹ The actual collagen binding domain is thus located between residues 911 and 1,365.⁷¹

These data are in conflict with observations indicating that the tryptic 52/48-kd fragment between residue Val (449) and Lys (728) contains the collagen-binding domain.⁷² This

fragment is identical to the fragment containing the GpIb-binding domain.⁶⁶ Recently, a more precise localization was achieved in studies using cyanogen bromide (CNBr) peptides of vWF. Two CNBr-peptides, residues 542 through 622 and 948 through 998, inhibited vWF binding to collagen type III. These peptides also bound directly to collagen. A MAb inhibiting binding of vWF to collagen type III bound selectively to both CNBr-peptides. These peptides correspond to the repeat domains A1 and A3 and contain regions of amino acid sequence homology (region 597 through 621 and 969 through 992) that may be involved in binding of vWF to collagen.⁷³

OTHER FUNCTIONAL DOMAINS

vWF also binds to heparin.^{32,74,75} Studies with *S. Aureus* V8 protease fragments have indicated that this binding domain is located in the amino-terminal fragment (SpIII).³² Recent data have further identified the binding domain⁷⁶ as being localized on the same 52/48-kd fragment between Val (449) and Lys (728), which contain the GpIb⁶⁶ and a collagen⁷² binding domain.

With regard to the domain involved in the binding of vWF to the matrix of cultured cells, less is known. Blotting studies with a MAb specifically blocking the binding of vWF to the matrix indicate that the domain resides on a carboxy-terminal 80-kd fragment of vWF, but no further data are available because the interaction of the MAb with this fragment was no longer demonstrable after reduction.²⁹

Little is known about the domain involved in the binding of FVIII coagulant protein to vWF. On FVIII, the binding site has been localized to the 80-kd light chain.⁷⁷ vWAgII, which is present in ~1% of the vWF chains, has been suggested to represent the binding site.³⁸ This was based on the stoichiometry of the FVIII-vWF interaction indicating that one molecule of FVIII is present per 100 vWF subunits. That uncleaved vWAgII would represent the binding site is unlikely, however, because vWF may be loaded with 12 to 15 times the normal amount of FVIII⁷⁸; this would not be possible if vWAgII were the only binding domain.

RELATION BETWEEN THE REPEATING DOMAINS IN vWF AND THE FUNCTIONAL SITES

The combined data about the internal homologies in vWF^{41,43} and the functional domains is summarized in Fig 2. No evidence indicates a close relationship between both types of domain structure. The GpIb domain is located on one A domain, but it is not repeated on the others, and the RGD sequence present in C₁ is not found in C₂. However, the two potential binding sites for collagen are both associated with the presence of A domains (A₁ and A₃). It will be of interest to see whether the exon-intron structure of vWF will correspond to the internal homologies and/or distribution of functional sites along vWF.

ROLE OF CARBOHYDRATE MOIETY IN vWF FUNCTION

Several authors have investigated the role of the carbohydrate side chains, in particular that of the sialic acid and

penultimate galactose residues in the structure and function of vWF. Studies performed some years ago first demonstrated that asialo-vWF obtained after neuraminidase treatment supported platelet aggregation independently of ristocetin when added to platelet-rich plasma.⁷⁹⁻⁸¹

This aggregation occurred through spontaneous binding of asialo-vWF to the platelet surface.⁸² More recently, using MAbs to GpIb-IIIa, two groups have proposed a mechanism.^{83,84} In a first step, asialo-vWF spontaneously binds to GpIb; this induces platelet activation and release as well as GpIb-IIIa exposure at the membrane surface. In a second step, in the presence of calcium, asialo-vWF and fibrinogen competitively bind to the exposed GpIb-IIIa receptor. Finally, binding of fibrinogen to GpIb-IIIa induces platelet aggregation. Intact vWF may substitute for fibrinogen in secondary binding to GpIb-IIIa, as demonstrated in patients with congenital afibrinogenemia.⁸⁵

However, in vivo, the role of asialo-vWF in platelet-platelet interaction by such a mechanism independent of any agonist remains speculative even though the release of a platelet neuraminidase following platelet aggregation by thrombin or collagen was recently described.⁸⁶

Platelet aggregation in a shear field may be induced by large multimers, particularly by exceptionally large forms found in the supernatant of cultured human endothelial cells.⁸⁷ Such forms have been observed in the plasma of patients with thrombotic thrombocytopenic purpura.⁸⁸

The role of the penultimate galactose in maintaining the multimeric structure and the ristocetin-cofactor activity of vWF is a subject of controversy. Treatment with neuraminidase followed by β -galactosidase or galactose oxidase was first reported to induce a drastic alteration of the multimeric structure of vWF⁸⁹ and a concomitant decrease of both the ristocetin-cofactor activity^{89,93} and the ability to bind platelets in the presence of ristocetin.⁹³ However, Federici et al⁹⁴ recently demonstrated that there was no loss of large multimers or ristocetin-cofactor activity after such enzymatic treatment of vWF, provided that protease inhibitors were present.

These data, which appear consistent with the finding that a small deglycosylated vWF fragment still interacts with GpIb in the presence of ristocetin,⁶⁶ suggest that the carbohydrate moiety of vWF may act as a potent protector against proteolytic degradation of the native protein.

CONCLUSIONS

vWF is one of the largest proteins in the body, both as a single subunit and certainly as a multimeric series. Recent advances in amino acid sequencing and molecular cloning have given insight into its composition. Functional studies have shown that the essential information for the function of vWF is present in its subunit 270-kd chain. Studies with proteolytic fragments using binding assays and specific MAbs have begun to unravel the relationship between the structure and function of this highly complex adhesive protein. At least five separate binding domains have been identified, of which four have been localized to some extent.

A rapid increase in understanding this intriguing protein may be expected in the near future.

NOTE ADDED IN PROOF

Using tryptic and plasmic digestion of vWF, the F.VIII binding domain has been localized on a 34 kD fragment of the N-terminal

end of the subunit,⁹⁵ starting at residue 1⁵¹ and extending to residue 272.⁹⁶

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Bipotent Cell Differentiation of KU-812: Evidence of a Hybrid Cell Line That Differentiates Into Basophils and Macrophage-Like Cells

By Takeaki Fukuda, Kenji Kishi, Yoshihisa Ohnishi, and Akira Shibata

KU-812-F is a subclone of KU-812, which has cytological features similar to the parent clone, and which carries the Philadelphia chromosome. We studied the effects of various chemical agents and serum-free culture (SF-C) condition on its differentiation. The KU-812-F subclone differentiated into macrophage-like cells with phorbol myristate acetate (PMA), but not with other agents. Unexpectedly, under SF-C, KU-812-F cells differentiated into mature basophil-like cells, and the histamine content of cell lysates increased in proportion to maturation. The addition of the condensed supernatant of SF-C promoted the cloning efficiency of KU-812-F cells in semisolid SF-C, whereas the cloning efficiency was reduced in SF-C alone. KU-812-F cells expressed myelomonocytic antigens. Additionally, My4 was induced with PMA and SF-C, but less often.

ACUTE MYELOGENOUS LEUKEMIA (AML) is known as a hematopoietic disease involving the cessation of differentiation of blastic cells in various stages of differentiation. On the other hand, chronic myelogenous leukemia (CML) represents an uncontrollable proliferation of leukemic cells with sequential maturation. However, the blastic crisis of CML (CML-BC) is biologically similar to AML. Despite these facts, the leukemic cells of some patients or some established leukemic cell lines derived from AML or CML differentiate to mature forms in vitro culture with or without various chemical compounds.¹⁻³ For example, HL-60, a well-known granulocytic cell line established from a patient with acute promyelocytic leukemia,⁴ differentiates into macrophage-like cells or mature neutrophils with phorbol ester⁵ or retinoic acid⁶ and dimethyl sulfoxide,⁷ respectively. K562 is another well-known granulocytic cell line established from a patient in CML-BC and has the capacity to synthesize hemoglobin when induced with hemin.^{8,9} These results indicate that leukemic cells may also differentiate partially or terminally with optimal stimulation.

Kishi established a unique Ph¹ positive leukemic cell line, designated KU-812, which had basophilic characteristics, and reported some cytological and immunological properties.¹⁰ In this report, we will describe the effects of various compounds or culture conditions on differentiation and the

HLA-DR was not expressed in any culture conditions, despite blastic morphology of KU-812-F cells or macrophage-like cells induced with PMA. The IgE receptor could not be demonstrated under any conditions. Correspondingly, immature and mature KU-812-F cells did not respond to anti-IgE or concanavalin A (con A), while histamine release was induced with PMA. In conclusion, KU-812-F belongs to the myeloid cell lineage and is at least a bipotent cell that can differentiate into basophils and macrophage-like cells. Although the functions appear to be dissimilar to those of normal basophils, the KU-812-F cell line may be a good model for basophil differentiation. Moreover, KU-812-F cells may provide new insights because they grow in semisolid culture by an autostimulating mechanism.

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biological characteristics of induced and noninduced KU-812-F.

MATERIALS AND METHODS

Cell. In this study, KU-812-F was used. KU-812-F is a subclone isolated by semi-solid culture of the parent clone KU-812 using 1.0% methylcellulose.¹⁰ The subclone possesses cytological properties similar to those of the parent KU-812. KU-812-F has been maintained in RPMI 1640 (Nissui, Tokyo) supplemented with 10% fetal bovine serum (FBS) (IBN, Takasaki, Japan), 50 IU/mL penicillin G, and 50 µg/mL streptomycin for over 1 year.

Chemical compounds. Dimethyl sulfoxide (DMSO) (WAKO, Tokyo), phorbol myristate acetate (PMA) (Sigma, St Louis), and retinoic acid (RA) (Sigma) were used as chemical inducers in optimal concentrations as described previously.⁵⁻⁷ GCT conditioned medium was obtained from GIBCO (Grand Island, NY) and used as colony-stimulating factor (CSF).

Morphology and Cytochemistry. Every two or three days a small amount of sample was taken for morphologic and cytochemical observations. Cytocentrifuged smears were prepared by Shandon type II cytopspin. Smears were stained with May-Grünwald-Giemsa stain and various other stainings were performed: myeloperoxidase (MPO), eosinophil peroxidase (EPO),¹¹ alpha-naphthyl butylate esterase (BE) with or without inhibition with sodium fluoride (NaF), naphthol-AS-D-chloroacetate esterase (ChE) with or without inhibition with NaF, aminopeptidase (Amp), acid phosphatase (AcP), alkaline phosphatase (AIP), β-glucuronidase (β-G), periodic acid-Schiff reaction (PAS) with or without diastase digestion, toluidine blue (TB), astra blue (AB), and alcian blue-safranin (AS). Different fixatives were also used simultaneously for TB and AB: iso-osmotic solution containing 0.6% formaldehyde and 0.5% acetic acid, Carnoy's solution, and ethanol-acetate (3:1).

Electron microscopic observation. Cell pellets were fixed in 2% phosphate-buffered glutaraldehyde for two hours, followed by postfixation with 1% phosphate-buffered osmium tetroxide for one hour. Specimens were dehydrated in graded ethanols, dealcoholized with propylene oxide, and embedded in EPON 812. Ultrathin sections were made, followed by double staining with uranyl acetate and lead citrate; they were observed with a Hitachi H-800 electron microscope (Hitachi Ltd, Tokyo).

Analysis of surface antigens. Analyses of surface antigens under various conditions were carried out using a cell sorter or an indirect immunofluorescence method. Antibodies used are listed in Table 1.

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Table 1. Surface Phenotype of KU-812-F Cells in Standard, Serum-free, and PMA Containing Culture Conditions

Antibodies	Percentages of Positive Cells		
	Standard	Serum-free (3 wk later)	PMA (3 d later)
Leu1	0-5	0-5	0-5
Leu2a	0-5	0-5	0-5
Leu3a + 3b	0-5	0-5	0-5
Leu4	0-5	0-5	0-5
Leu7	0-5	0-5	0-5
Leu11b	0-5	0-5	0-5
Leu12	0-5	0-5	0-5
CALLA	0-5	0-5	0-5
HLA-DR	0-5	0-5	0-5
Transferrin	90-100	90-100	80-90
LeuM1	0-5	0-5	0-5
HPCA-1	0-5	0-5	0-5
OKT3	0-5	0-5	0-5
OKT4	0-5	0-5	0-5
OKT6	0-5	0-5	0-5
OKT8	0-5	0-5	0-5
OKT10	0-5	0-5	0-5
OKT11	0-5	0-5	0-5
OKIa	0-5	0-5	0-5
OKM1	80-90	50-65	75-85
My4	10-15	30-45	45-55
My7	85-100	90-100	80-90
My9	90-95	90-100	90-100
Mo1	75-85	40-50	70-80
B1	0-5	0-5	0-5
B2	0-5	0-5	0-5
PCA-1	0-5	0-5	0-5
BA1	0-5	0-5	0-5
BA2	0-5	0-5	0-5
BA3	0-5	0-5	0-5
3A1	0-5	0-5	0-5
H107	0-5	0-5	0-5

Induction of differentiation. DMSO, PMA, and RA in optimal concentrations were added to each culture. GCT-conditioned medium was mixed with culture medium in various concentrations from 5% to 20%. The cells with each inducer were cultured in Iscove's modified Dulbecco's medium (IMDM) (Boehringer-Mannheim, West Germany) supplemented with 10% FBS, 10 mL/L sodium pyruvate (GIBCO), 10 mL/L nonessential amino acids (GIBCO), 50 IU/mL penicillin G, and 50 µg/mL streptomycin. Under serum-free conditions (SF-C), KU-812-F cells were cultured with IMDM supplemented with 5 µg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 50 IU/mL penicillin G, 50 µg/mL streptomycin, 10 mL/L sodium pyruvate, and 10 mL/L nonessential amino acids. Each culture was harvested in a humidified atmosphere of 5% CO₂ at 37°C. Half of the medium in each culture was replaced with freshly prepared medium every three days. Every two or three days a small amount of sample was taken for morphological, cytochemical, and immunological analyses. Cell viability was determined by the trypan blue dye exclusion test.

Clonal growth of KU-812-F in semi-solid culture. KU-812-F cells were cultured in semi-solid medium under serum-supplemented and -free conditions. KU-812-F cells were mixed in IMDM containing 0.3% agar (Difco, Detroit) and 10% FBS or insulin and transferrin. KU-812-F cells were added to give final concentrations of 10,000, 1,000, and 100 cells per milliliter. A 1-mL cell suspension

was placed on a 35 × 10 mm plastic dish (Corning Glass Works, Corning, NY). Colonies (>10 cells) were scored under an inverted microscope (Nikon, Tokyo) after seven days of incubation. Smears of cells from each colony were prepared by cytopsin and stained with May-Grünwald-Giemsa or MPO. In addition, as a preliminary examination, we used 25-fold concentrated conditioned medium to determine whether the supernatant contained growth and/or differentiation factor(s). The conditioned medium (SF-CM) was prepared from the supernatant of KU-812-F cells cultured in SF-C by using Minicon (B15; Amicon, Danvers, MA). SF-CM was added to several dishes at a concentration of 10%. We also tested the effects of CSF and interleukin-2 (IL-2, Genzyme Co, Boston). CSF was added to each culture at concentrations of 5%, 10%, and 20%, respectively. IL-2 was mixed to final concentrations of 20, 50, and 100 IU/mL, respectively.

Histamine content. Every five days the cells in SF-C were resuspended in Hanks' balanced salt solution and washed three times. The washed cells were resuspended in 2.5 mL of the same solution with 2% peroxide chloride for 30 minutes at room temperature. Cell debris was removed and cell lysates were used for fluorometric detection of histamine as Siraganian described previously.¹² The cells in standard and PMA-containing cultures were also used for detecting histamine. K562 and HL-60 cells were used as controls.

Degranulation response to chemical mediators. Degranulation of immature and mature KU-812-F cells was assessed by detection of histamine in the supernatants after incubation with chemical mediators. Briefly, immature and mature KU-812-F cells were incubated with purified IgE (10⁵ cells in 1 mL with 40 µg IgE) (IgE, Serotec Ltd, Blackthorn, Bicester, England) for 30 minutes at 37°C, followed by addition to each culture of concanavalin A (con A, 20 µg/mL) or optimally diluted monoclonal mouse anti-human IgE (Medical and Biological Laboratories, Nagoya, Japan). The cultures were harvested after ten minutes at 37°C. Controls consisted of the same KU-812-F cells with IgE, con A, or anti-IgE alone. Moreover, PMA at 10⁻⁸ mol/mL was added to the cultures of immature or mature KU-812-F cells to detect release of histamine in the supernatant as described previously.¹³

Chromosome analysis. KU-812-F cells were treated with 0.075M KCl hypotonic solution for 30 minutes at 37°C, and fixed by methanol:acetate (3:1). Chromosomes were banded by the trypsin-Giemsa method.

RESULTS

Morphologic and cytochemical analysis of KU-812-F under various culture conditions. Cytochemical analyses of cells under various culture conditions are indicated in Table 2. In standard and CSF-added conditions, morphologic and cytochemical analyses of KU-812-F cells in various periods were identical (Fig 1), except for the presence of a small population of mature forms. A small amount of MPO positive cells was also observed. Reactivity of BE was completely inhibited with NaF. Positivity of each cell for PAS was markedly reduced after digestion with diastase. However, a fine granular positive reaction was still observed. Proliferation rates were moderately decreased in cultures with DMSO, RA, and PMA. KU-812-F cells cultured with DMSO and RA showed no apparent changes in morphology. PMA induced the differentiation of KU-812-F cells to macrophage-like cells in two to three days; these cells adhered to the culture bottles and showed ample vacuolated

Table 2. Cytochemical Analysis of KU-812-F Cells in Standard, Serum-free, and PMA Containing Culture Conditions

Days	Standard					Serum-free					PMA				
	0	5	10	15	20	0	5	10	15	20	0	5	10	15	20
MPO	0-5	0-5	0-5	0-5	0-5	0-5	0-5	0-5	5-10	5-10	0-5	0	0	0	0
EPO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BE	55-60	65-70	65-70	55-60	60-70	60-65	70-80	65-85	70-80	70-85	60-65	60-70	60-65	70-85	70-85
ChE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AcP	80-90	75-85	80-90	90-100	85-95	80-90	75-80	80-90	90-100	80-90	90-95	90-95	90-95	90-95	90-95
AlP	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AmP	40-50	45-50	35-45	50-55	45-50	45-50	40-45	50-55	55-60	55-60	35-45	40-40	35-40	30-40	25-35
β G	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95	90-95
PAS	85-90	80-90	85-90	90-95	90-95	80-85	90-95	90-95	95-100	95-100	80-90	70-75	60-65	50-60	50-55
TB	20-30	20-30	20-30	20-30	25-30	20-30	70-75	75-80	85-90	90-100	20-25	10-15	10-15	15-20	10-15
AB	10-15	20-25	15-20	10-15	10-15	10-15	45-55	60-75	80-90	80-95	10-15	10-15	5-10	5-10	5-10
AS*	10-15	10-15	15-20	10-15	10-15	10-15	25-30	40-50	55-65	70-80	10-15	0-5	5-10	0-5	0-5

Each range was determined by three independent experiments; percentages of positive cells were determined by counting more than two hundred cells.

*The cells with blue-staining granules were regarded as positive cells.

cytoplasm (Fig 2). As the number of macrophage-like cells increased, the percentage of positive cells for TB, AB, and AS decreased. These macrophage-like cells showed slight phagocytic activity. In SF-C, doubling time became slightly longer, and degenerative cells were increased over time. Unexpectedly, cytoplasmic granules in each cell and in the mature cells gradually increased (Fig 3). Over 60% of cells showed band or segmented configurations 3 weeks later (Table 3). In proportion, the numbers of cells positive for TB, AB, and AS increased considerably (Figs 4, 5). Cells positive for PAS increased markedly, compared with controls. Approximately 5% of the cells were positive for MPO, but negative for EPO. However, there was no evidence of increase of macrophage-like cells or cells showing adhesion or phagocytic activity. Immature or mature KU-812-F cells under various conditions were positive for TB and AB with different fixative procedures.

Electron microscopic observation. Noninduced KU-812-F cells revealed immature blastic configuration with or without specific granules as described by Kishi.¹⁰ Mature KU-812-F cells had segmented nuclei with dense clumping of chromatin along the nuclear membranes (Fig 6). Unlike noninduced KU-812-F cells, the mature forms possessed moderate amounts of microfilaments in the perinuclear area and more numerous specific granules. The peculiar granules had coarse granular matrix with or without myelinoid materials and glycogen particles (Fig 7). However, scrolls, whorls, or crystalloid structures, often present in mast cells, could not be observed. There was a moderate amount of glycogen particles in the cytoplasm.

Surface phenotype of KU-812-F cells in various culture conditions. Results of surface marker analyses are summarized in Table 1. In standard condition, over 95% of the KU-812-F cells were positive for My7, My9, OKM1, Mol, and anti-transferrin receptor. Surface antigens of KU-812-F cells cultured with CSF, DMSO, and RA showed no alteration. As the number of macrophage-like cells induced with PMA increased, the percentage of cells positive for My4 increased to 60% three days later. In SF-C, the positivity of My4 increased gradually. In contrast, the percentage of cells

positive for OKM1 and Mol decreased. The positivity for HLA-DR or Ia-like antigen was not increased. Three weeks later, 45% of the KU-812-F cells expressed My4, while 80% showed mature basophil-like configurations. This finding suggested that some basophil-like cells expressed My4. KU-812-F cells did not express the IgE receptor under any culture conditions.

Clonability of KU-812-F cells in semisolid culture. Results are summarized in Table 4. Cloning efficiency was strikingly decreased in 100 cells per milliliter cell concentration in each culture condition. In the relatively high cell concentration of 10,000 cells per milliliter, clonal efficiency ranged from 16% to 25% (mean, 18.6%) in standard culture. Cloning efficiency was markedly reduced in serum-free culture. CSF and IL-2 had no effect on cloning efficiency. In contrast, the addition of SF-CM increased cloning efficiency to 9 to 14 (mean, 13%), compared with SF condition. Despite decreased cloning efficiency, similar results were obtained in cell concentrations of 1,000 cells per milliliter. The morphology of each colony was similar to the original KU-812-F. The number of cells with characteristic granules increased slightly in SF and SF added with SF-CM conditions. Some colonies contained a few MPO positive cells admixed with MPO negative cells. Colonies comprising only MPO positive cells or only macrophage-like cells were not observed in each experiment.

Histamine content. Histamine content in cell lysates of KU-812-F cells in SF-C increased with length of culture (Table 5). However, histamine content did not increase under any other conditions. In controls, there were trace amounts of histamine.

Table 3. Differential Counts of KU-812-F Cells in Serum-free Culture

Duration	Blast	Pr.	Myel.	Met.	Band.	Seg.
Standard	80.5	13.5	0.5	1.0	1.5	3.0
Week 1	47.5	15.5	7.0	8.0	10.0	12.0
Week 2	18.5	15.5	13.0	10.5	7.5	35.0
Week 3	10.5	10.5	4.0	10.0	15.0	51.0

The results are expressed as the mean of three experiments.

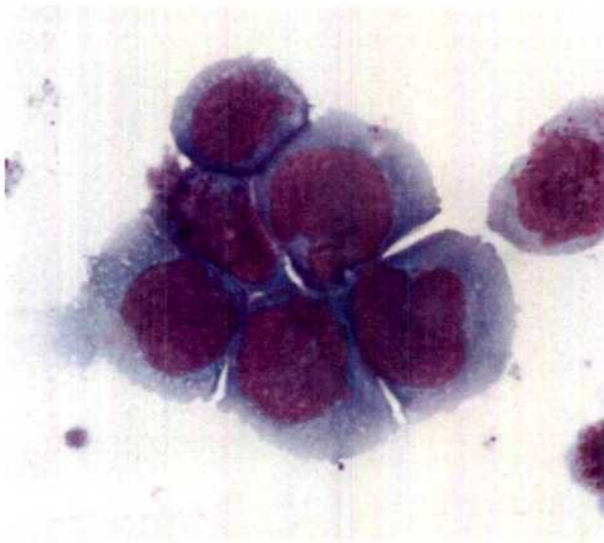


Fig 1

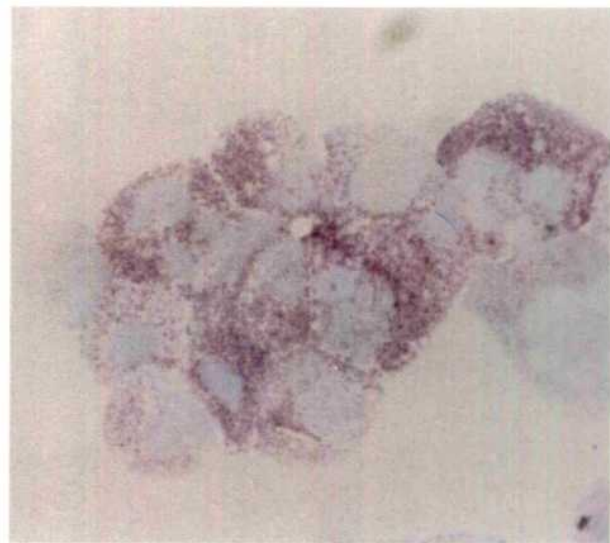


Fig 4

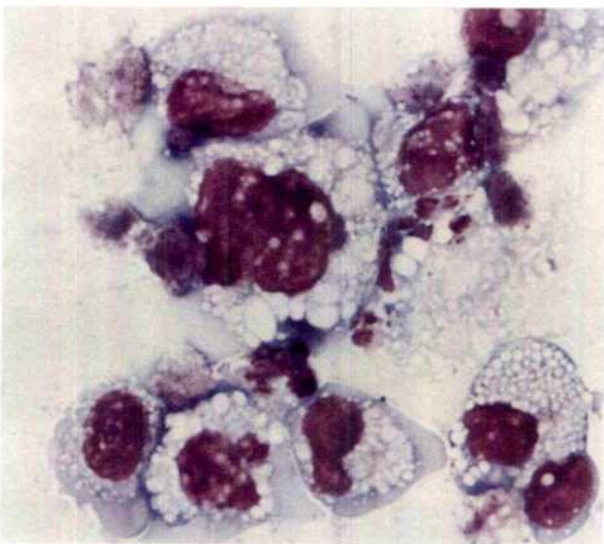


Fig 2

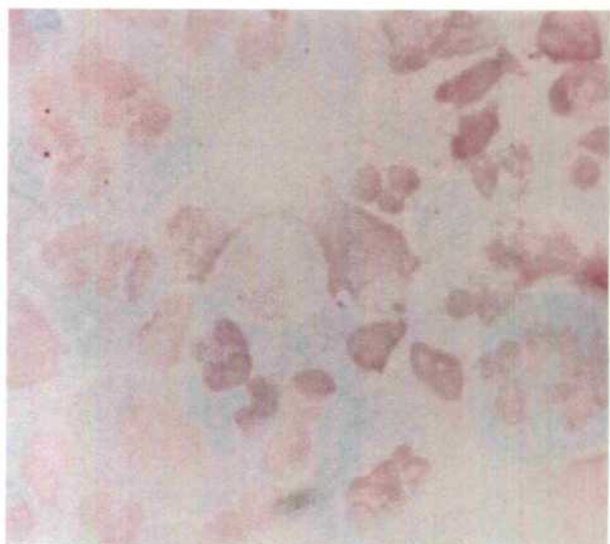


Fig 5

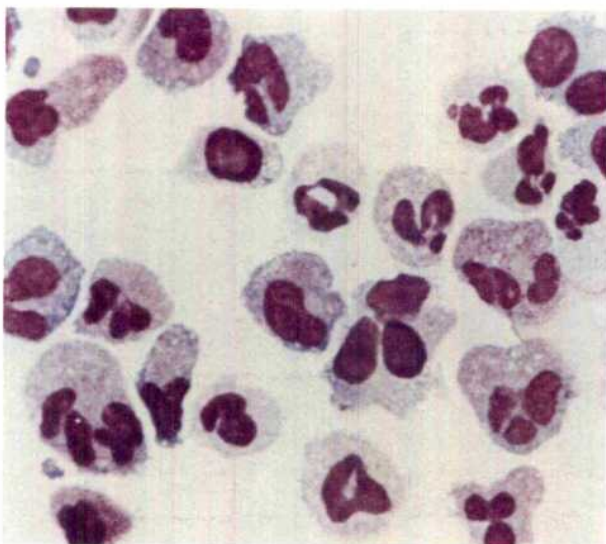


Fig 3

Fig 1. KU-812-F cells in standard condition reveal immature blastic appearance with or without granules (May-Grünwald-Giemsa stain). (Magnification $\times 650$.)

Fig 2. KU-812-F cells induced with PMA show macrophage-like cells possessing large vacuolated cytoplasm without granules (May-Grünwald-Giemsa stain). (Magnification $\times 650$.)

Fig 3. KU-812-F cells differentiate into mature basophil with a segmented nucleus and numerous fine cytoplasmic granules in serum-free culture in about three weeks (May-Grünwald-Giemsa stain). (Magnification $\times 650$.)

Fig 4. Almost all mature KU-812-F cells contain numerous fine granules with metachromasia (TB staining). (Magnification $\times 650$.)

Fig 5. The fine granules in mature KU-812-F cells are positive for AB. (Magnification $\times 650$.)

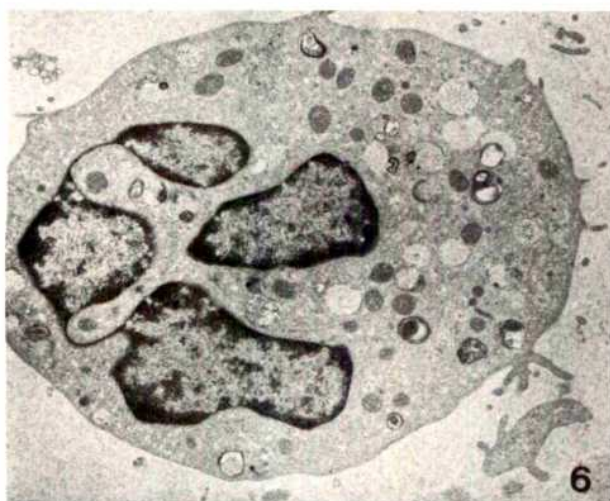


Fig 6. Ultrastructure of a mature KU-812-F cell shows a lobulated nucleus with dense chromatin clumping along the nuclear membrane, numerous characteristic granules, and a moderate amount of glycogen particles. (Original magnification $\times 4500$; current magnification $\times 3,870$.)

Histamine release activity. Anti-IgE or con A did not result in an increase of histamine in the supernatant of induced or noninduced KU-812-F cells. On the other hand, supernatant challenged with PMA contained more histamine than that of controls (Table 6).

Chromosome studies. Chromosomal analysis of a total of 30 cells revealed a hyperdiploid karyotype. Basic changes were similar to those previously described.¹⁰ Ph¹ chromosome was present in all karyotypes analyzed. An inversion or deletion of No. 16 was not present.

DISCUSSION

KU-812-F cells show basophilic characteristics under standard culture condition. Like HL-60,⁵ when stimulated with PMA, KU-812-F developed into macrophage-like cells with ample vacuolated cytoplasm, adhesion to culture bottles, loss of basophilic features, an increase of BE positive cells, a moderate increase of My4 expression, and slight



Fig 7. The specific granules are composed of coarse granular matrix with or without myelinoid materials, similar to typical basophil granules. (Original magnification $\times 12,500$; current magnification $\times 8,500$.)

Table 4. The Number of Colonies (>10 Cells) in Various Cell Concentrations in Standard, SF, and SF Added SF-CM Conditions

	100 cell/mL	1,000 cell/mL	10,000 cell/mL
Standard	4.8 ± 0.8	79.8 ± 4.9	$1,860.8 \pm 120.1$
SF	1.1 ± 0.6	10.8 ± 3.7	86.4 ± 20.5
SF-CM	3.4 ± 1.5	67.6 ± 3.6	$1,304.0 \pm 75.0$

Results are mean $\pm$ SD of colonies from five independent experiments.

phagocytic activity. Thus, KU-812-F can differentiate at least into basophils and macrophage-like cells. We have not yet observed other divergent differentiation of KU-812-F, such as into erythroid cells, megakaryocytes, neutrophils, or eosinophils, in any culture conditions.

CML is a hematopoietic disease involved at a pluripotent stem cell level.¹⁴⁻¹⁶ In most cases of CML-BC, blastic cells cease normal differentiation, but show limited differentiation along one cell lineage.¹⁷ Recently, it has been reported that blastic cells in CML-BC of some patients revealed mixed or multilineage cell differentiation.^{18,19} Ph¹ positive leukemic cell lines established from CML patients, such as K562 and BV173, also expressed multilineage differentiation.^{20,21} Moreover, recent advances in analysis of surface antigens using monoclonal antibodies or genes for immunoglobulins and T cell receptors indicate that lineage infidelity is occasionally observed in not only CML but also acute leukemias.^{22,23} Lineage infidelity of leukemic cells as indicated by surface phenotype or differentiation ability may be attributable to blocks at divergent stages of differentiation in hematopoiesis.^{24,25} Likewise, it is not surprising that KU-812-F cells differentiated into at least two cell lineages, because it is a Ph¹ positive leukemic cell line established from a patient with CML-BC.¹⁰

While differentiation of KU-812-F cells to basophils was not induced with any chemical agents used in this study, these cells unexpectedly differentiated into mature basophil-like cells under SF-C. Light and electron microscopic observation and increase of histamine in cell lysates indicated apparent basophilic differentiation of KU-812-F in SF-C. In culture conditions with neither insulin nor transferrin, or medium alone, proliferation of KU-812-F cells was strikingly reduced in a short time. Therefore, both insulin and transferrin are essential to the growth of KU-812-F cells in SF-C, but do not promote their differentiation. The presence of a small number of mature basophil-like cells in standard culture and the apparent maturation of KU-812-F cells in SF-C led us to hypothesize that the KU-812-F clone itself produces some soluble factor(s) promoting its growth and differentiation. High cloning efficiency of KU-812-F cells in relatively high cell concentrations also appeared to support this hypothesis. Our preliminary examination also revealed that SF-CM promoted the cloning efficiency of KU-812-F cells. Autostimulation has also been found in other human leukemic cell lines.²⁶⁻²⁸ Moreover, Chung et al recently demonstrated that CSF was produced by murine mast cell lines.²⁹ Therefore, it is likely that KU-812-F cells secrete some soluble factor(s). However, the substance(s) contained in SF-CM seems to be a growth but not a differentiation factor(s). SF-CM differs from at least GCT-CM and IL-2

Table 5. Histamine Content of KU-812-F Cells Under Various Culture Conditions (ng/mL/10⁶ cells)

Days	0	5	10	15	20
Standard	35.7 ± 4.7	41.0 ± 3.0	39.8 ± 2.9	33.1 ± 1.8	32.5 ± 2.2
Serum-free	39.7 ± 2.4	42.2 ± 1.8	80.8 ± 6.0	186.8 ± 17.8	252.2 ± 10.2
PMA	30.4 ± 0.9	11.4 ± 1.6	8.6 ± 1.3	—	—
K562	trace	trace	trace	trace	trace
HL-60	trace	trace	trace	trace	trace

The results are expressed as the mean ± SD of three experiments.

because these substances did not promote clonability of KU-812-F cells. A more detailed examination will be required to determine whether a differentiation factor is present in SF-CM. These results are preliminary and we are attempting to separate this factor(s) from SF-CM. On the other hand, Lozzio et al demonstrated that K562 cells differentiated along the granulocytic lineage under poorly nutritional medium.²⁰ Hozumi showed that K562 cells differentiated into monocytoic cells with actinomycin D or PMA after culture in SF-C.³⁰ Fischkoff et al also postulated that nutrient depletion played an important role in the induction of HL-60 cells into eosinophils.³¹ Thus, SF-C or nutrient deprivation may provide suitable stimuli for differentiation of some leukemic cells. Autostimulation and nutritional deletion may facilitate the differentiation of KU-812-F cells into basophils.

Parkin et al demonstrated that some blastic cells had basophilic features in lymphoid as well as myeloid crisis of CML.³² Parkin et al and Polli et al also reported that some lymphoid blasts were positive for platelet peroxidase and had basophilic granules.^{32,33} These findings lead us to suggest that basophilic precursors may be regarded as lymphoid or other type blasts in some cases. Wick et al also pointed out that AML with basophilic differentiation was occasionally misdiagnosed as lymphocytic leukemia.³⁴ Determination of surface phenotype of basophilic precursors may provide a parameter for subtyping leukemic blastic cells. Pruzansky and Patterson described the presence of myeloid surface markers on normal basophils.³⁵ Ishizaka reported that human basophils were positive for OKT3, OKM1, and Ia-like antigen.³⁶ A few cases of basophilic crisis of CML have been reported with accompanying analysis of surface markers with monoclonal antibodies.^{37,38} Fukuda demonstrated that leukemic basophil precursors expressed My7, My9, and OKM1, but no HLA-DR or Ia-like antigen, even

in blastic configuration.³⁹ As illustrated in Table 1, KU-812-F cells expressed My7, My9, OKM1, and Mol in standard culture; mature forms expressed similar surface markers under SF-C. Therefore, in addition to the ultrastructural demonstration of basophilic granules, the expression of granulomonocytic antigens, such as My7, My9, or OKM1 etc, should be considered for phenotyping of basophil progenitors.

It is said that myeloid precursors also express HLA-DR or Ia-like antigen on their surfaces.^{40,41} KU-812-F cells did not express these antigens under any culture conditions, even on the surface of macrophage-like cells induced with PMA. Similarly, HLA-DR or Ia-like antigens were not detected on the basophil precursors of basophilic crisis in CML.³⁷⁻³⁹ These facts indicate that the precursor of the human basophil has no HLA-DR or Ia-like antigens or has lost them in some stage of the neoplastic process. Further study will be needed to confirm this conclusion.

The addition of PMA to KU-812-F cells resulted in the expression of My4, suggesting monocytic differentiation. Under SF-C, the percentage of cells expressing My4 was greater than that in standard conditions and the percentage expressing OKM1 or Mol decreased. These phenotypic changes, as well as the presence of a small number of MPO positive cells, suggest that the KU-812-F clone consists of two different clones or a hybrid clone. Failure to detect colonies comprising exclusively MPO positive cells or monocytoic cells seems to argue against the possibility of the presence of two different clones. The finding that My4 positivity was higher than that of MPO and overlapped with TB, AB, or AS positive rates is also indicative of My4 expression on some TB, AB, and AS positive cells. Moreover, Griffin et al demonstrated that HL-60, when induced to differentiate to neutrophils with DMSO, expressed My4 as well as other myeloid antigens.⁴² Therefore, KU-812-F appears to be a hybrid cell line.

Recent studies using semisolid cultures suggested a close relationship between basophils and eosinophils. Although basophils form mixed colonies with other hematopoietic cells, they tend to occur in colonies admixed with eosinophils.⁴³⁻⁴⁵ This finding suggests that basophils and eosinophils may derive from a common progenitor.⁴⁶ However, morphologically identified eosinophils were not detected and EPO was negative in KU-812-F cells under any conditions. A normal chromosome No. 16 suggested no association with eosinophils in the KU-812-F subline, because the characteristic abnormality of chromosome No. 16 was present in acute myelomonocytic leukemia with abnormal eosinophils or HL-60 cells showing eosinophilic features.^{31,47} Leary et al provided information more suggestive of the nature of KU-

Table 6. Histamine Content in the Supernatant of Immature or Mature KU-812-F Cells Challenged by Chemical Mediators (ng/mL/10⁶ cells)

Mediators	Histamine Content
Anti-IgE	
Immature	0.6 ± 0.2
Mature	1.2 ± 0.3
Con A	
Immature	0.7 ± 0.1
Mature	2.2 ± 0.3
PMA	
Immature	43.9 ± 4.0
Mature	188.6 ± 16.4

The results are expressed as the mean ± SD of three experiments.

812-F because they found a colony that comprised both basophils and macrophages.⁴⁵

Although the cytochemical and functional similarities of both basophils and mast cells have been recognized, the exact origin of or relationship between the two cells remains obscure. Zucker-Franklin found the intermediate cells that possessed a mixture of granules characteristic of basophils as well as mast cells and proposed a common precursor of the two cells.⁴⁸ However, we did not observe these intermediate cells in induced or noninduced KU-812-F cells. Ultrastructurally, KU-812-F cells, especially the mature forms, could be distinguished from mast cells by their multilobulated nuclei and characteristic granules with a coarse granular matrix.^{49,50} The findings of a negative reaction for ChE and histamine release in response to PMA argue against their being mast cells.⁵¹⁻⁵³ Furthermore, KU-812-F cells expressed myeloid markers, while mast cells did not.⁵⁴ On the basis of these findings, maturation of KU-812 cells in SF-C seems to occur along basophil but not mast cell lineage. It remains to be seen whether KU-812-F cells develop into mast cells under some specific environments.

One of the particular characteristics of the basophil is the presence of the IgE receptor on its surface. Abnormal functions of basophils derived from CML in chronic or

accelerated phases have been reported.^{52,55} Although we analyzed the IgE receptor in induced and noninduced KU-812-F cells using monoclonal antibody for the IgE receptor H107,⁵⁶ we failed to detect or induce it. No histamine release was seen in immature and mature KU-812-F cells in response to anti-IgE or con A; this may reflect an abnormal expression of the IgE receptor. The abnormal function of KU-812-F cells and basophils originating from CML may be due in part to the abnormal expression of the IgE receptor. Some possibilities are postulated; first, the number of IgE receptors is too small to detect by immunofluorescence; second, the lack of expression of the IgE receptor on the surface of KU-812-F cells may be due to an unknown mechanism during the neoplastic process; third, a defective IgE receptor with a structure extremely distinct from the normal may be involved, and therefore, IgE cannot bind to it. Abnormal expression of the IgE receptor may be due to a genetic defect not yet identified.

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Patients With a Prolonged Bleeding Time and Normal Aggregation Tests May Have Storage Pool Deficiency: Studies on One Hundred Six Patients

By H. Karel Nieuwenhuis, Jan-Willem N. Akkerman, and Jan J. Sixma

One hundred six patients with storage pool deficiency (SPD) were studied with respect to platelet count, bleeding time, total platelet ATP and ADP, platelet serotonin, and in vitro aggregation. The diagnosis of SPD was made on basis of a prolonged bleeding time, a decreased total platelet ADP, and a diminished level of serotonin. Fifty-one patients from 34 unrelated families had congenital SPD, and 55 patients had acquired SPD. Congenital SPD was a common disorder in patients with a lifelong bleeding tendency and a prolonged bleeding time. The frequency in this group of

patients was 18%, about one-half the frequency of von Willebrand's disease (vWd). Twenty-three percent of all patients had normal aggregation responses to ADP, epinephrine, and collagen; 33% had aggregation tracings typical for a secretion defect; and 44% had miscellaneous aggregation abnormalities. These findings indicate that SPD is common, heterogeneous, and not necessarily associated with in vitro aggregation abnormalities.

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THE BLEEDING TIME is one of the most important screening tests in patients with a bleeding tendency.¹ A prolonged bleeding time in association with a normal platelet count points to von Willebrand's disease (vWd) or a platelet function disorder. Appropriate investigations of the von Willebrand factor (vWf) complex identify vWd, and aggregation tests are the first routine diagnostic tools to identify a platelet function disorder. In many patients, however, this approach fails to explain the bleeding tendency because no aggregation abnormality is found.

In 1980, we studied a patient with a prolonged bleeding time and normal aggregation tracings. At first, storage pool deficiency (SPD) was excluded because of the normal aggregation tracings. Further investigations, however, disclosed decreased levels of platelet nucleotides and serotonin, consistent with SPD. This observation prompted us to determine platelet nucleotides and serotonin in all patients with an unexplained prolonged bleeding time, independent of the results of the aggregation tests.

This article describes our experiences and reports that patients with a prolonged bleeding time and normal aggregation tests may have hereditary or acquired SPD.

PATIENTS AND METHODS

With informed consent of the patients and control subjects, venous blood was collected into 0.1 vol of 135 mmol/L of trisodium citrate. Platelet aggregation studies were performed at 37°C in a Payton Dual Channel Aggregation Module (Payton Associates, Buffalo) or a PAP-4 aggregometer (Bio/Data, Hatboro, PA) with 2.5 and 5 μ mol/L of ADP (DADE Diagnostics, Aquada, Puerto Rico), 1.0 and 5 μ mol/L of epinephrine (DADE Diagnostics), 1.0 and 4.0 μ g/mL of equine collagen (Hormon Chemie, Munich), 1.5 mmol/L of arachidonic acid (Bio/Data) and 10 μ mol Ionophore A23187 (Boehringer, Mannheim, FRG). The platelet number, as measured

with the Platelet Analyzer 810 (Baker Diagnostics, Bethlehem, PA) was adjusted to 250,000/ μ L by dilution with autologous platelet-poor plasma (PPP).

The results of the aggregation tracings were classified into three categories: (a) normal response to ADP, epinephrine, and collagen; (b) "typical" pattern of a secretion disorder: absence of second wave in response to low-dose and high-dose ADP or epinephrine, little aggregation response to low-dose collagen and normal or decreased aggregation response to high-dose collagen; and (c) all other miscellaneous defects.

The contents of the dense granules were investigated by measuring total ATP and ADP in ethanol extracts with the luciferin-luciferase technique² and by determination of endogenous serotonin.³ The bleeding time was measured with a Simplate II device (General Diagnostics, Morris Plains, NJ) using a venous pressure of 40 mm Hg and a horizontal incision on the volar surface of the forearm.

The diagnosis of vWd was made on the basis of a prolonged bleeding time and decreased levels of VIII:C, vWf:Ag and vWf:ristocetin cofactor, or an abnormal mobility of vWf in crossed immunoelectrophoresis. If a diagnosis could not be made at first examination, investigations of the vWf parameters were repeated twice.

One hundred six patients with SPD were studied. They fulfilled the following three criteria for storage pool deficiency: (a) prolonged bleeding time, (b) diminished level of total platelet ADP, and (c) decreased platelet serotonin. We excluded 15 patients, who were studied in the same period, because they fulfilled only two criteria: Seven patients, with myeloproliferative syndrome, had normal bleeding times and abnormal levels of platelet nucleotides and serotonin; eight patients had prolonged bleeding times and abnormal levels of platelet nucleotides but normal values for platelet serotonin.

All patients with congenital SPD were subjects referred because of a bleeding tendency. They were studied at least twice (mean 2.3, range 2 through 8). The diagnosis of congenital SPD was made in 10 patients before 1980. They were reinvestigated for the present study. In the other patients, the diagnosis of congenital or acquired SPD was made between January 1980 and August 1986. Patients with acquired SPD were studied because of a bleeding tendency or for preoperative screening. The results of aggregation studies in four patients with acquired SPD were excluded from the analysis because of low platelet counts (67,000 to 90,000/ μ L). The other patients had platelet counts >100,000/ μ L. Nineteen patients have been subjects of previous studies on various aspects of SPD.^{4,6}

The patients with congenital SPD and the control subjects had not ingested any drugs for at least 2 weeks prior to the study. The patients with acquired SPD had not used drugs known to influence platelet function. For control purposes, healthy volunteers were accepted from among laboratory and medical personnel (mean age 31 years, range 19 to 57 years; men 32, women 31).

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RESULTS

Patients. The diagnosis of SPD was made in 106 patients (62 women, 44 men) on the basis of a prolonged bleeding time, a decreased level of total ADP, and decreased platelet serotonin (Fig 1). Fifty-one patients (30 women, 21 men) from 34 unrelated families had congenital SPD. In 7 patients (2 families), the defect was part of the Hermansky-Pudlak syndrome. In one family, the defect was associated with another congenital abnormality: SPD was found in a boy with severe hemophilia B and in his sister who was a carrier for hemophilia B. In 9 patients (6 families), congenital SPD was associated with mild thrombocytopenia (100,000 to 140,000 platelets/ μ L). In the other patients, the platelet disorder was the sole abnormality.

Fifty-five patients had acquired SPD. The diagnoses of the associated clinical disorders are given in Table 1.

Aggregation tests. ADP 5 μ mol/L, epinephrine 1.0 μ mol/L, and collagen 1.0 μ g/mL induced irreversible aggregation tracings in 19 of 20 control subjects, with a maximal change in light transmission ranging from 50% to 100%. The pattern of aggregation in these 19 subjects was the reference for the classification of category A. If the patients' platelets did not respond to low concentrations of the agonists, higher concentrations of collagen (4 μ g/mL) and epinephrine (5 μ mol/L) were also tested.

At first presentation, 13 patients with congenital SPD and 10 patients with acquired SPD had normal aggregation responses to ADP, epinephrine, and collagen (category A). Seventeen patients with congenital SPD and 17 patients with acquired SPD had a typical pattern of a secretion disorder (category B). Twenty-one patients with congenital and 24 patients with acquired SPD had atypical aggregation tracings (category C). All kinds of patterns were found in this category, eg, normal ADP aggregation with decreased epinephrine- and collagen-induced aggregation, abnormal

Table 1. Patients With SPD

SPD	No. of Patients
Congenital	51
Acquired	55
Acute nonlymphocytic leukemia	5
Acute lymphocytic leukemia	3
Myelodysplastic syndrome	8
Chronic granulocytic leukemia	7
Chronic myelomonocytic leukemia	1
Polycythemia vera	4
Essential thrombocythemia	6
Myelofibrosis	2
Chronic lymphocytic leukemia	2
Hodgkin's disease	1
Waldenstrom's macroglobulinemia	1
Paroxysmal nocturnal hemoglobinuria	2
Erythropoietic protoporphyria	1
Systemic lupus erythematosus and SLE-like disease	7
Endocarditis	1
Disseminated intravascular coagulation	4

Abbreviation: SLE, systemic lupus erythematosus.

ADP- and epinephrine-induced aggregation with normal response to low-dose collagen, decreased epinephrine-induced aggregation with normal ADP- and collagen-induced aggregation, or abnormal aggregation in response to a low dose of collagen with a normal response to ADP. The overall frequency of the different aggregation types at first presentation in 102 patients studied was: category A, 23%; category B, 33%, and group C, 44%.

To study the variability of the aggregation tests in congenital SPD, we compared the tracings of the first and second investigations. A consistent pattern of aggregation on repeated study was found in 11 of 13 patients (85%) in category A and in 11 of 17 patients (65%) in category B. Inconsistency among the aggregation studies was commonly found in category C. Only 9 of 21 patients (43%) were again classified in category C.

The parameters used in this study to diagnose SPD were also variable on repeated examinations. The bleeding time was performed 154 times in 51 patients with congenital SPD. A normal bleeding time was found 14 times. Total ADP and serotonin, both measured 117 times in these patients, were in the normal range 6 times and 20 times, respectively.

Normal aggregation tracings. The first patient with normal aggregation tests and SPD was found in 1980. She had a lifelong bleeding tendency and a prolonged bleeding time of 22 minutes. She was studied eight times over a period of 5 years, and the normal aggregation tracings and platelet abnormalities (platelet nucleotides, serotonin, bleeding time) were consistent. Deficiency of the dense granules in this patient was further confirmed by direct measurement of ATP and ADP in the granule compartment by digitonin-induced cell lysis as described previously.⁶

In two unrelated families, two members of each family had the combination of SPD, prolonged bleeding time, and normal aggregation studies.

All 17 patients of category A on whom the tests were performed had a normal response to arachidonic acid and ionophore A23187.

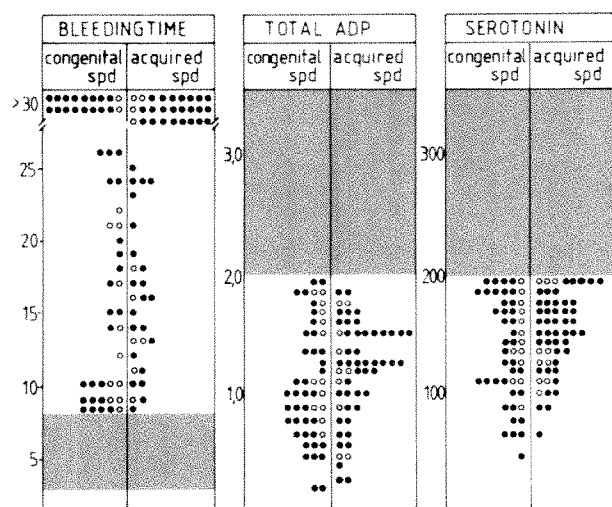


Fig 1. Bleeding time (expressed in minutes), total ADP (in μ mol/ 10^{11} platelets) and serotonin (in nmol/ 10^{11} platelets) in 106 patients with storage pool deficiency. Shaded areas are the normal ranges (mean $\pm$ 2 SD), which for bleeding time is 5.6 ± 2.6 (n = 61), for total ADP 4.0 ± 2.0 (n = 63), and for serotonin 370 ± 170 (n = 15). Patients with normal aggregation tests (O).

After 1980, we determined platelet nucleotides and serotonin in every patient with an unexplained prolonged bleeding time independent of the results of the aggregation tests. Excluding the results of patients diagnosed before 1980, we calculated that the frequency of normal aggregation tests in patients with congenital SPD is 31% (13 of 41 patients); in patients with acquired SPD is 20% (10 of 50 patients).

Relative frequency of congenital SPD. To define the relative frequency of congenital SPD, we reviewed the medical records of all 390 patients referred to our outpatient clinic for a bleeding tendency between January 1982 and December 1985. None of the patients had ingested drugs, nor did they have another disorder. A prolonged bleeding time associated with a normal platelet count was observed in 145 patients. In this group of 145 patients, vWd was diagnosed in 52 patients (36%), congenital SPD was diagnosed in 27 (18%), defect of thromboxane synthesis was diagnosed in 4 (3%), and platelet function disorders with miscellaneous aggregation abnormalities (secretion defect, defect of response to thromboxane A₂, or other poorly defined aggregation abnormalities) was diagnosed in 23 (16%). We found no explanation for the bleeding tendency associated with a prolonged bleeding time in 39 patients (27%).

DISCUSSION

Patients with congenital or acquired SPD have a mild bleeding disorder associated with a prolonged bleeding time⁷⁻¹⁰; their platelets are deficient in the nonmetabolic pool of ADP, which is stored in the dense bodies.^{6,11,12} We studied 106 patients with SPD; 23% of the patients had normal aggregation tracings.

These data provide a perspective on aggregation studies in SPD that differs completely from that of many previous reports and reviews.¹³⁻¹⁵ It is widely held that SPD platelets show a characteristic pattern of aggregation on stimulation in vitro: absence of second wave of aggregation with ADP and epinephrine and impaired response with collagen. In contrast, we found this pattern in only 33% of patients. The other patients had either normal aggregation responses or biphasic responses to at least one of the agonists. The high frequency of normal aggregation tests in this study may be a consequence of our approach to patients with a bleeding tendency and a prolonged bleeding time; assays for platelet nucleotides and serotonin were performed independently of the results of the aggregation tests.

In this study, we defined the pattern of the irreversible aggregation tracing with a change of light transmission of >50% as "normal." This definition implies that one can sometimes observe "abnormal" aggregation tests in a normal population; in this study, one of 20 subjects, and in a previous study, 8 of 80 controls had reversible tracings.¹⁶ If one were also to classify these patterns as normal, even more SPD patients may be classified as normal aggregation tests.

To evaluate the aggregation tests in a well-defined group of patients, we required abnormalities of all three parameters (bleeding time, total ADP, and serotonin) on the first examination for the diagnosis of SPD. The variability of the laboratory findings in the patients with congenital SPD indicates, however, that more patients with a mild bleeding

disorder may have SPD. Repeated testing may be required to establish the diagnosis SPD in these subjects. The same problem was described by Abildgaard and colleagues¹⁷ in the diagnosis of vWd.

Normal aggregation tracings or minimal aggregation abnormalities have been described previously in a few patients with congenital SPD,¹⁸⁻²¹ but not in acquired SPD. Lages and Weiss¹⁸ reported two patients with SPD in whom biphasic aggregation responses to ADP and epinephrine consistently were observed. Platelets from these patients secreted greater quantities of ADP after stimulation by epinephrine than did SPD platelets with comparable ADP deficiencies and absent second-phase aggregation. In addition, their platelets were extremely sensitive to stimulation with ADP. These two mechanisms and endoperoxide- or thromboxane-mediated aggregation could contribute to the biphasic aggregation.

In the present study, we observed normal aggregation tracings in patients with excessively long bleeding times and manifest bleeding symptoms, indicating that in many patients no correlation exists between in vitro aggregation and the in vivo situation. We found no correlation between the categories A, B, and C and clinical manifestations of bleeding.

We conclude that SPD is common, is heterogeneous, and is not necessarily associated with in vitro aggregation abnormalities. Thus, SPD cannot be excluded with simple aggregation studies. These findings will change the approach to the bleeding patient with a prolonged bleeding time.

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High Serum Interleukin-2 Receptor Levels Are Related to Advanced Disease and a Poor Outcome in Childhood Non-Hodgkin's Lymphoma

By Ching-Hon Pui, Stephen H. Ip, Patrick Kung, Richard K. Dodge, Costan W. Berard, William M. Crist, and Sharon B. Murphy

The clinical usefulness of serum interleukin-2 receptor (IL2R) measurements was determined in 59 children with non-Hodgkin's lymphoma (NHL) and six with B cell acute lymphoblastic leukemia (B-ALL). Levels of the receptor showed a clear relationship to disease stage, as follows: B-ALL > stage III or IV diffuse small noncleaved-cell NHL > stage III or IV lymphoblastic NHL > stage I or II NHL. Soluble IL2R levels were also closely correlated with serum lactic dehydrogenase levels ($r = 0.7$, $P = .0001$), a recognized indicator of tumor cell burden. Children with higher soluble

IL2R levels ($>1,000$ U/mL) were significantly more likely to fail treatment ($P = .001$), even when the analysis was limited to those with more advanced disease: stages III and IV NHL and B-ALL ($P = .02$). In a multivariate analysis, soluble IL2R level was found to have greater predictive strength than either serum lactic dehydrogenase level or disease stage. Thus, the measurement of soluble IL2R in children with NHL could be expected to improve existing methods of risk assignment in this disease.

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INTERLEUKIN 2, originally termed T cell growth factor, is synthesized and secreted by antigen- or lectin-activated T lymphocytes in the presence of macrophage-derived interleukin-1 and must interact with specific high-affinity membrane receptors to exert its biological effects.^{1,2} The interleukin-2 receptor (IL2R or Tac antigen) is not present on the surface of resting T or B lymphocytes but is rapidly expressed following activation.³⁻⁵ Membrane IL2R has also been found on certain B or T cell malignancies including Burkitt's lymphoma,⁵ hairy cell leukemia,^{5,6} and human T-cell leukemia virus (HTLV)-I-associated adult T cell leukemia.⁷

Using monoclonal antibodies directed against different epitopes of human IL2R, Rubin et al⁸ detected a soluble form of IL2R that is released by activated normal peripheral blood mononuclear cells and synthesized in large amounts in vitro by HTLV-I-infected leukemic cell lines. The soluble IL2R is smaller than its cellular counterpart but retains the ability to bind interleukin 2. Subsequent studies disclosed comparable levels of soluble IL2R in cord blood and peripheral blood from normal adults.^{9,10} Increased serum levels of IL2R have been found in patients with certain B or T cell malignancies, including HTLV-I-associated adult T cell leukemia, Sézary syndrome, Hodgkin's disease, chronic lymphocytic leukemia, and hairy cell leukemia.⁹

Because of the role of IL2R in T and B cell proliferation and the possibility that serum levels of the receptor influence

responsiveness to chemotherapy,⁹ we measured soluble IL2R in a relatively large group of children with non-Hodgkin's lymphoma (NHL) or B cell acute lymphoblastic leukemia (B-ALL). The receptor was present in serum samples from all patients, was found in higher levels in those with more advanced disease, and proved to have independent prognostic significance.

MATERIALS AND METHODS

Patients. From 1979 to 1986, 99 consecutive children with NHL or B-ALL were admitted to three clinical trials¹¹⁻¹³ at St Jude Children's Research Hospital, depending on the histologic features and stage of their disease. Serum samples taken before the start of chemotherapy were available for 65 of these patients. The 41 boys and 18 girls with NHL ranged in age from 1.8 to 17.9 years (median, 10.7 years). The three boys and three girls with B-ALL were 2.3 to 10.2 years old (median, 5.8 years). In each case the diagnosis was based on a combination of clinical, anatomic, histologic, and cytologic criteria, with addition of immunologic and cytogenetic studies in some instances. The Working Formulation¹⁴ was used to classify cases into diffuse small noncleaved-cell, lymphoblastic, or large cell (noncleaved, cleaved, and immunoblastic) types. The first category encompasses not only Burkitt's tumor but also lymphomas that have been designated as undifferentiated non-Burkitt's type. The diagnosis of B-ALL was made from the presence of surface immunoglobulins on bone marrow blast cells with L3 morphology according to French-American-British (FAB) criteria.¹⁵

A stage was assigned to each case of NHL with use of a previously described system.¹⁶ Children with localized NHL in favorable sites have stage I or II disease. Stage III includes disseminated disease on both sides of the diaphragm, extensive unresectable intraabdominal disease, and all primary epidural or anterior mediastinal tumors without bone marrow or CNS involvement. Stage IV is defined by initial CNS and/or bone marrow involvement ($\leq 25\%$ blast cells) in addition to other tumor sites. Cases with $>25\%$ malignant B cells in the bone marrow were classified as B-ALL, representing advanced B cell NHL in a phase of leukemic evolution. In this study, 30 patients had diffuse small noncleaved-cell NHL (stage I in eight, II in ten, III in 11, and IV in one); 22 had lymphoblastic NHL (stage I in one, II in two, III in 14, and IV in five); seven had large cell NHL (stage I in three, and II in four) and six had B-ALL.

Soluble IL2R was also measured in 12 children with otitis media (OM) who were otherwise normal and ranged in age from 1 to 6 years (median, 3 years).

Treatment. Children with stage I or II NHL were treated according to a Pediatric Oncology Group (POG) protocol that uses three cycles of cyclophosphamide, doxorubicin, vincristine, and prednisone for remission induction and consolidation therapy and 6

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months of 6-mercaptopurine and methotrexate for continuation therapy.¹¹ Children with stage III or IV small noncleaved-cell NHL or B-ALL received 6 months of intensive treatment with alternating courses of either high-dose fractionated cyclophosphamide followed by vincristine and doxorubicin or coordinated high-dose methotrexate and cytarabine.¹² Patients with stage III or IV lymphoblastic NHL were treated on a protocol designed for high-risk ALL in which teniposide plus cytarabine was added to an otherwise conventional regimen of therapy.¹³ Informed consent was obtained for all patients, and the investigation was approved by the institution's clinical trials committee.

Determination of soluble IL2R. Soluble IL2R was measured with a sandwich enzyme immunoassay available as the CELLFREE Interleukin-2 Receptor Test Kit (T-cell Science, Inc, Cambridge, MA). In brief, a mouse monoclonal antibody to human IL2R was first adsorbed onto a polystyrene microtiter well. A patient's sample or standard was added to the antibody-coated well and unreacted sample components were removed by washing. A second horseradish peroxidase-conjugated murine monoclonal antibody to human IL2R was then used to bind a second epitope on the molecule captured by the first antibody. After removal of unbound enzyme-conjugated anti-IL2R by washing, a substrate solution was added to the well. After terminating the reaction by a stop solution, the absorbance at 490 nm was measured. The average value of absorbance from duplicate samples was then plotted on a standard curve and converted to a numerical value. Serum levels of IL2R are expressed in units per milliliter. A reference preparation of 1,000 U/mL of supernatant from phytohemagglutinin-stimulated peripheral blood lymphocytes was used as a standard. The normal serum IL2R values in healthy adult donors range from 50 to 500 U/mL (mean, 260 U/mL).

Determination of serum lactic dehydrogenase. The total activity of serum lactic dehydrogenase (LDH) was measured with the Monitor Kinetic AMB-610 assay on the KDA analyzer (American

Monitor Corp, Indianapolis). Samples with enzyme activities >700 U/L were diluted and reassayed; the values obtained were then multiplied by the dilution factor. The normal values for our laboratory range from 30 to 300 U/L.

Statistical analysis. The Kruskal-Wallis test was used to compare soluble IL2R or serum LDH levels among different subgroups of patients with NHL. The Pearson product-moment correlation (r) and t test analyses were used to determine the association between soluble IL2R and serum LDH levels. Time-to-failure curves were constructed by the Kaplan-Meier procedure¹⁷ with differences analyzed by the log-rank test.¹⁸ Time to failure was defined as the interval between achievement of remission and relapse or death due to any cause. Patients who did not enter remission were assigned a failure time of zero. The influence of potentially significant prognostic factors on time to failure was estimated with the Cox proportional-hazards model,¹⁹ which permits comparison of treatment outcome for two or more subsets of patients while simultaneously adjusting for the effect of other factors (covariates) in the model. Final selection of factors for the model depended on whether or not the P value was <.10 after adjustment for other variables already in the model, using a forward stepwise procedure. An IL2R value of 1,000 U/mL was chosen as the dividing point between high and low receptor levels because it coincided well with clinically distinct groups of diseases and treatment outcome.

RESULTS

All patients had detectable soluble IL2R levels (Fig 1), including the 12 children with OM (404 to 942 U/mL; median, 615 U/mL). The highest values were found in sera from patients with B-ALL (1,030 to 17,725 U/mL; median, 3,283 U/mL). Children with stage III or IV small noncleaved-cell NHL had soluble IL2R levels of 329 to 5,335 U/mL (median, 1,832 U/mL), significantly higher than the

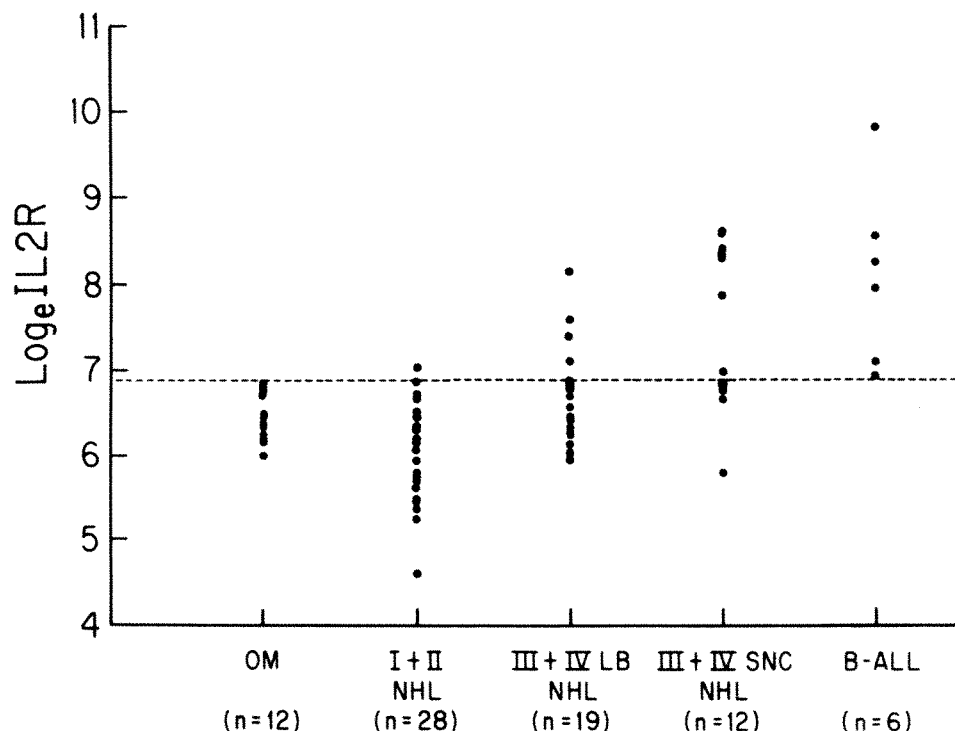


Fig 1. Distribution of serum IL2R levels among children with OM; stage I or II NHL; stage III or IV lymphoblastic (LB) NHL; stage III or IV diffuse small noncleaved-cell (SNC) NHL; and B-ALL. The dashed line separates IL2R values above or below 1,000 U/mL.

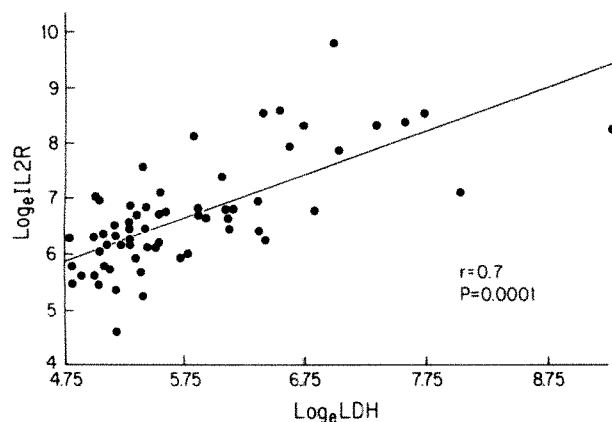


Fig 2. Comparison of log serum IL2R levels and log serum LDH levels. The straight line represents the least-squares regression fit to the data.

376 to 3,390 U/mL (median, 808 U/mL) for patients with stage III or IV lymphoblastic NHL ($P = .02$). The latter concentrations were in turn greater than those found in stage I or II NHL, 100 to 1,143 U/mL (median, 477 U/mL; $P = .001$). Only one of the 28 subjects with low-stage disease had a soluble IL2R level above 1,000 U/mL. The distribution of serum LDH levels among the various subgroups of patients showed the same pattern as was noted for IL2R: B-ALL > stage III or IV diffuse small noncleaved-cell NHL > stage III or IV lymphoblastic NHL > stage I or II NHL (data not shown). There was, in fact, a strong positive

correlation between serum IL2R and LDH levels in this study (Fig 2).

Higher soluble IL2R levels were associated with a poorer treatment outcome. Nine of 18 patients with levels above 1,000 U/mL, compared with only six of 47 with lower levels, have failed therapy (Fig 3A, $P = .001$). Even when patients with stage I or II disease were excluded, high levels of soluble IL2R were still associated with a poor outcome (Fig 3B, $P = .02$). Because of the possible interrelationships among clinical and biologic risk factors in our patients, a Cox regression analysis was used to assess the relative importance of each factor after adjustment for the effects of other covariates. As shown in Table 1, both soluble IL2R level and serum LDH level were found to have independent prognostic value.

DISCUSSION

The most reliable prognostic factors in childhood NHL have been the stage of disease at diagnosis and serum LDH level.^{12,16,20,21} We report here that high soluble IL2R levels predict a poor treatment outcome even after adjustment for these two factors. The increased serum IL2R levels in our patients could reflect greater release of the receptor from either malignant cells or activated normal lymphocytes. We favor the first explanation because soluble IL2R levels not only correlated with disease stage but also showed a linear relationship with serum LDH levels, a reliable indicator of the total body burden of malignant cells in both ALL²² and NHL.^{12,20,21}

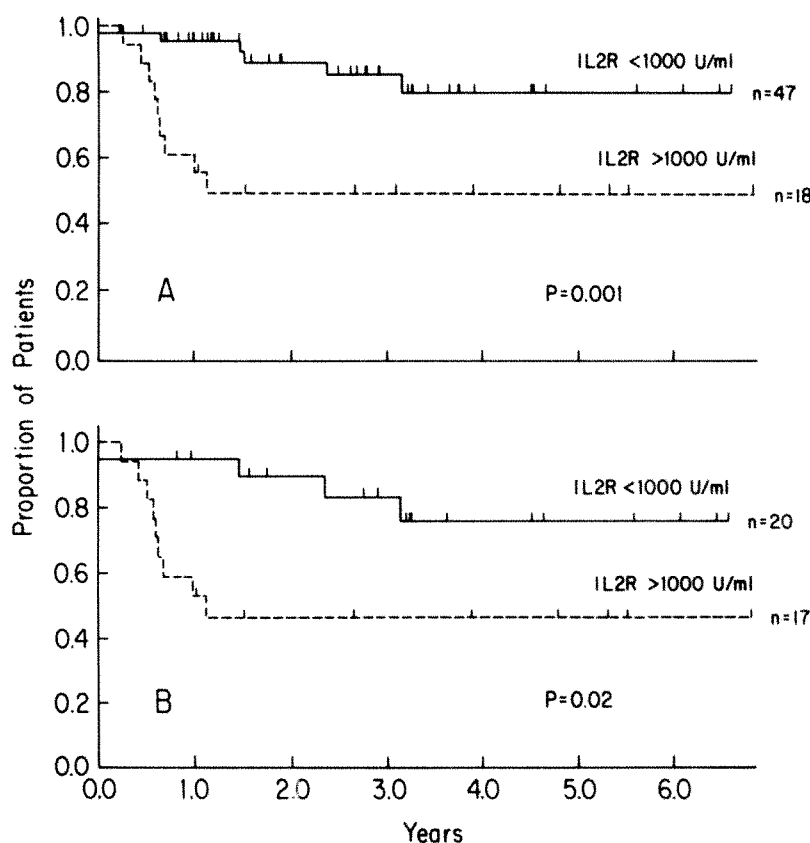


Fig 3. Comparison of time-to-failure rates according to IL2R for (A) all patients with NHL and B-ALL; (B) patients with stage III or IV NHL or B-ALL. Significantly worse treatment results were evident for patients with higher levels (>1,000 U/mL) in both comparisons.

Table 1. Relationship of Serum IL2R Level and Other Presenting Features to Time to Failure in 65 Children With NHL or B-ALL

Feature	Category		P Values*
	Better	Worse	
Univariate analysis†			
IL2R level (U/mL)	< 1000	> 1000	.004
LDH level (U/L)	≤300	>300	.007
Stage	I, II	III, IV, or B-ALL	.017
Histologic type	Lymphoblastic	Diffuse small noncleaved cell	.65
Multivariate model‡			
IL2R level (U/mL)	< 1000	> 1000	.004
LDH level (U/L)	≤300	> 300	.065
Stage	I, II	III, IV, or B-ALL	.62
Histologic type	Lymphoblastic	Diffuse small noncleaved cell	.48

*From the likelihood ratio test.

†Comparison of time to failure, with use of the Cox proportional-hazards model, between patients with the better v worse risk feature for relapse in the indicated category, without adjustment for the effects of other variables.

‡As above, except that stepwise regression analysis was used to identify the best predictors of treatment outcome, taking into account the competing effects of all covariates entered in the Cox model.

The function of cellular IL2R in lymphoid malignancies has not been fully elucidated. Several cases of common, pre-B or T cell ALL have been induced to express IL2R after in vitro activation²³⁻²⁵ and, in some cases, interleukin 2 stimulated subsequent colony formation of neoplastic progenitor cells in vitro.^{23,24} Leukemia cells from some patients with T cell chronic lymphocytic leukemia were shown to have

the receptors and a good proliferative response to exogenous interleukin 2.^{26,27} However, HTLV-1 associated adult T cell leukemia constitutively expressed high levels of cell surface IL2R but had no (or very poor) proliferative responses to interleukin 2.^{26,28} Even less is known about the functional significance of soluble IL2R. Since soluble IL2R is capable of binding interleukin 2,⁸ it may have an immunoregulatory role by competing with cellular IL2R for the ligand and thus down-regulating the immune response. In this regard, the soluble IL2R has been suggested to be a "blocking factor" produced by the malignant cells to inhibit the host's immune response to the tumor.^{8,9}

Children with OM were found to have elevated soluble IL2R levels compared with that reported for healthy adults.⁹ In fact, their levels were higher than those of patients with stage I or II NHL ($P = .02$). These increased levels were probably the result of lymphocyte activation in response to infection. In view of this finding, one could question the usefulness of soluble IL2R levels as indicators of residual tumor, as suggested by Rubin et al.⁸

We conclude that the level of soluble IL2R in children with NHL has independent prognostic significance, higher levels being associated with more advanced disease, greater tumor burden, and a poorer outcome. Understanding the biological significance of soluble IL2R will assume increased importance with wider use of biologic response modifiers in cancer therapy.

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Monoclonal Antibody Ki-B3 Detects a Formalin Resistant Antigen on Normal and Neoplastic B Cells

By Alfred C. Feller, Hans H. Wacker, Gerhard Moldenhauer, Heinz J. Radzun, and Mohammad R. Parwaresch

A new monoclonal antibody Ki-B3 produced by a fusion with leukemic cells of a centroblastic/centrocytic lymphoma (m.l. follicular) is introduced. This antibody predominantly recognizes B cells of follicular mantle and germinal center cells, as well as plasma cells in normal lymphoid tissue. Furthermore, 80% of all low- and high-grade B cell lymphomas are stained, whereas among T cell lymphomas, only four of 15 T lymphoblastic lymphomas were positive to Ki-B3. All peripheral T cell lymphomas showed a negative reaction. Additionally, Ki-B3 detects a small percentage of monocytes and some myelomonocytic leukemias. All

epithelial tissues as well as all sarcomas tested were invariably negative. Ki-B3 precipitates a 220 kiloDalton (kD) molecular weight antigen similar to the leukocyte common antigen. Presumably Ki-B3 detects a subtype of the leukocyte common antigen that is predominantly expressed on mature and immature B cells. As the antigen is formalin resistant, Ki-B3 can be used in routine hematology on paraffin sections for the detection and differential diagnosis of B cell lymphomas.

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IMMUNOHISTOLOGICAL TECHNIQUES have been of considerable value in the diagnosis and classification of reactive and neoplastic diseases. The introduction of monoclonal antibodies (MoAbs) recognizing lymphoid cell surface antigens or epithelial membrane antigens has opened new perspectives.¹⁻⁵ Different phenotypic properties may also be associated with different clinical prognoses.⁶ However, the use of such MoAbs is limited by the way of tissue preparation and fixation, since most antigens recognized by MoAbs are denatured or masked in their antibody binding site by formalin fixation.⁷ For this reason, it is necessary to use frozen tissue sections for immunolabeling. This encompasses the major disadvantage that the tissue must be frozen immediately after biopsy, stored at -70°C , and fixed in a manner that does not allow for a reliable morphological evaluation of the section.⁸ Today, only a limited number of MoAbs are available that recognize antigens after formalin fixation.⁹⁻¹² These are MoAbs directed against common leukocyte and epithelial antigens or S100 protein. Besides two MoAbs, —LN1 and LN2—that stain B cell antigens after B5 fixation,¹³ to our knowledge there are no others available with the property to detect formalin resistant antigens on cells of lymphoid subsets.

In this article we report on the production of a new MoAb, Ki-B3, that recognizes a formalin resistant antigen expressed on normal and neoplastic mature and immature human B cells.

MATERIALS AND METHODS

Antibody production. The monoclonal antibody Ki-B3 was generated according to the method described by Köhler and Milstein.¹⁴ BALB/c mice were immunized three times in 2-week intervals with 5×10^6 leukemic cells from a patient with centroblastic/centrocytic lymphoma.^{15,16} Two weeks after the last dose, animals were boosted with 5×10^6 cells applied four, three, and two days before fusion. Equal numbers of spleen cells were fused with mouse myeloma cell line X63-Ag 8.653. Hybridomas were selected in HAT medium and cloned by limited dilution repeatedly.¹⁷ High-titer MoAb was raised as ascites in pristane primed mice.

Specificity testing. Culture supernatants were tested immunohistochemically on cryostat sections of normal human tonsils and cytospin preparations of the leukemic cells. The reaction was performed using immunoperoxidase method.¹⁸ Bone marrow smears were stained with an immunoalkaline phosphatase method.¹⁹ For immunostaining of two antigens the immunoalkaline phosphatase

technique using two MoAbs and two different couplers as described elsewhere were applied.²⁰

Additionally, normal human tissue samples of skin, brain, muscle, heart, lung, salivary gland, thyroid gland, stomach, colon, intestine, liver, gall bladder, pancreas, kidney, adrenals, urinary bladder, prostate gland, testis, uterus and ovary, as well as lymphatic and hematopoietic tissue (Table 1) were tested with Ki-B3. Human leukocyte fractions were obtained by gradient centrifugation²¹ of defibrinated blood of normal volunteers ($n = 5$). Monocytes were separated by glass adherence.²² Lymphocytes were incubated with neuraminidase-treated sheep erythrocytes²³ in order to separate B lymphocytes from rosetting T lymphocytes by gradient centrifugation. Granulocytes were purified from the pellet after gradient centrifugation. Purity control of all cellular fractions was performed by immunocytochemistry using MoAbs listed in Table 2. Peritoneal macrophages were obtained from serous effusions during diagnostic pelviscopy. Bone marrow cells were investigated on fresh smears from hematologically healthy patients.

Routinely processed material was fixed in 4% formalin, paraffin embedded, and 5 μm paraffin sections were performed. Material from 58 carcinomas, 44 sarcomas, 19 malignant melanomas, and five neuroendocrine carcinomas was investigated. Ninety cases of low-grade and 87 cases of high-grade malignant B cell lymphomas were added. The study included 40 cases of peripheral and 15 cases of lymphoblastic T cell lymphomas. T lymphoblastic lymphomas have been phenotyped with a large panel of MoAbs detailed elsewhere.²⁴ Malignant lymphomas were tested on cryostat section as well as on formalin-fixed paraffin-embedded material. All lymphoma cases were diagnosed in the Kiel Lymphoma Registry by light microscopy in hematoxylin-eosin (H&E) and Giemsa stained paraffin sections. The diagnosis and cellular origin was proven by immunohistochemistry using an immunoperoxidase method on frozen sections, with MoAbs listed in Table 3. We hereby declare

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Table 1. Reactivity of Ki-B3 With Lymphatic Tissues and Normal Bone Marrow Cells

Lymphatic Tissue	Ki-B3
Lymph node	
Germinal center (%)	50-60
Follicular mantle (%)	50-80
Primary follicle (%)	50-60
T zone	Single cells positive
Interdigitating	Negative
Reticulum cells	
Dendritic reticulum	Negative
Cells	
Plasma cells	Positive
Thymic gland	
Medulla	Single cells positive
Cortex	Negative
Spleen	
White pulp	B areas positive
Red pulp	Single cells positive
Bone marrow	
Myeloid	Single monocytes positive
Erythroid	Negative
Megakaryocytes	Negative

Table 2. Results of Purity Control of Various Cell Fractions Using MoAb Including Ki-B3. Counts (Mean $\pm$ SD) Are Given in Percent of Positive Cells

Cell Fractions	MoAbs				
	Ki-B3	To15	Leu-4	VIM-D5	Ki-M1
Granulocytes	—	1 $\pm$ 2	2 $\pm$ 2	95 $\pm$ 4	ND
Monocytes	3 $\pm$ 2	3 $\pm$ 2	12 $\pm$ 4	ND	82 $\pm$ 9
T lymphocytes	2 $\pm$ 1	4 $\pm$ 2	95 $\pm$ 3	ND	ND
B lymphocytes	91 $\pm$ 6	93 $\pm$ 5	5 $\pm$ 4	ND	ND
Peritoneal	—	ND	ND	98 $\pm$ 2	97 $\pm$ 3
Macrophages	—	ND	ND	ND	ND
Platelets	—	ND	ND	ND	ND

Abbreviation: ND, not done.

Table 3. Specification of Various MoAb Used to Specify Lymphomas, Carcinomas, and Sarcomas, Investigated

MoAb	Cluster	Specificity	Source/Reference
C leukocyte	CD45	All leukocytes	Dako, Hamburg
HMFG2		Human milk fat globulin	Bics, Munic
To15	CD22	All B cells	Dako
HD39	CD22	All B cells	Behring Werke AG, Marburg
Lyt3	CD2	Sheep erythrocyte receptor	New England Nuclear
Anti-Leu4	CD3	All T cells	Becton Dickinson (BD)
Anti-Leu1	CD5	All T cells	BD
		Some B cells	
Anti-Leu6	CD1	Thymic cortex cells	BD
VIL-A1	CD10	Common ALL antigen	Knapp et al ²⁵
VIM-D5	CD15	Granulocytes	Majdic et al ²⁶
Ki-M1	CD11c	Monocytes, macrophages	Radzun et al ²⁷
Anti-IgM		IgM	BD
Anti-IgD		IgD	BD
Anti-kappa		Ig light chain kappa	Dianova, Hamburg
Anti-lambda		Ig light chain lambda	Dianova, Hamburg
Ki-M6*		Monocytes, macrophages	Parwaresch et al ²⁸
Ki-M8*		Monocytes, macrophages	Radzun et al ²⁹
MY7*	CD13	Granulocytes, monocytes	Coulter, Krefeld
MY9*	CD33	Monocytes, myelocytes	Coulter, Krefeld
3C4*	CD15	X hapten	Schientle et al ³⁰

*MoAbs especially used for phenotyping myeloid and myelomonocytic leukemias.

that all blood samples were obtained with the informed written consent of each patient exclusively for diagnostic purposes.

Additionally, 25 cases of acute myeloid or myelomonocytic leukemia were investigated on cytospin preparations using the MoAbs listed in Table 3. The lymphoid origin could be excluded by the negativity to CD3, CD5, CD10, and CD22 antibodies. All leukemias were primarily acute leukemias but not blast crisis.

All tissue preparations were stained with an immunoperoxidase method using the MoAbs described in this paper (Ki-B3). Parallel sections of carcinomas and sarcomas as well as of malignant lymphomas were subjected to an immunostaining with a common leukocyte antibody (common leukocyte; DAKO, Hamburg) and an epithelial membrane antibody (HMFG2; Bios, Munich).

Before immunostaining, all paraffin sections were deparaffinized with xylene for 20 minutes and trypsinized (trypsin EC 3.4.21.4; Sigma, Munich, FRG) for another 30 minutes. Trypsinization was performed before incubation with all MoAbs except for Ki-B3. One hundred milligrams of trypsin was dissolved in 100 mL Tris-NaCl.

Cell surface iodination and immunoprecipitation. Cells of the human B lymphoblastic line LICR-LON-HMY-2 (kindly provided by the London Branch of the Ludwig Institute of Cancer Research, Sutton, UK) were radio-iodinated by the lactoperoxidase method essentially as described by Goding.³¹ 10^7 cells (viability >95%) were labeled with 0.5 mCi ¹²⁵Iodide (Amersham-Buchler, Braunschweig, FRG) and subsequently solubilized in lysis buffer containing 1% Nonidet P-40 and proteinase inhibitors (10 mmol/L phenylmethylsulfonylfluoride and 20 mU/mL aprotinin). The cell lysate was precleared by precipitation with affinity-purified rabbit anti-mouse Ig and Protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). The glycoprotein fraction was isolated by a lens culinaris lectin-Sepharose 4B (Pharmacia) column. For immunoprecipitation cell lysate or glycoproteins of 1 to 2×10^7 cells were mixed with 50 μ L 20-fold concentrated MoAb supernatant, 15 g rabbit anti-mouse Ig, and 20 μ L Protein A-Sepharose 4B (50% saturation). After overnight incubation at 4°C with gentle rotation, the adsorbent was washed extensively and then boiled in reducing sodium dodecylsulfate (SDS) sample buffer. Samples were subjected to polyacrylamide slab gel electrophoresis (SDS-PAGE) using a discontinuous buffer system.

RESULTS

Ki-B3: Immunohistology. A total of 176 supernatants of the primary cultures were tested. From these, 152 showed a negative reaction to tonsils and leukemic lymphoma cells. Twenty-one supernatants showed strong reactivity with different tissue compartments, ie, lymphoid cells, collagen fibres, and muscular tissue. Only three culture supernatants showed a restricted reactivity to the leukemic lymphoma cells and to lymphoid cells of tonsillar tissue. These supernatants were additionally tested on formalin-fixed and paraffin-embedded material of human tonsil. Only one supernatant (Ki-B3) showed a positive reaction that was identical to the reaction pattern on cryostat sections. Ascites samples

were tested in different dilutions before staining of different tissue types. The final dilution for cryostat as well as for paraffin section was 1:5,000 diluted in phosphate buffered saline.

Ki-B3 stained more than 95% of cells in cytospin preparations of leukemic blood cells of the patient with centroblastic/centrocytic (CB/CC) lymphoma. In human tonsils this MoAb stained 50% to 80% of follicular mantle cells and about 50% to 60% of cells in germinal centers predominantly in the light zone (Fig 1). About 50% to 60% of cells in primary follicles showed a positive reaction to Ki-B3. In the T cell areas and interfollicular areas only some single cells were positive. Plasma cells localized in the subepithelial connective tissue revealed a strong reactivity.

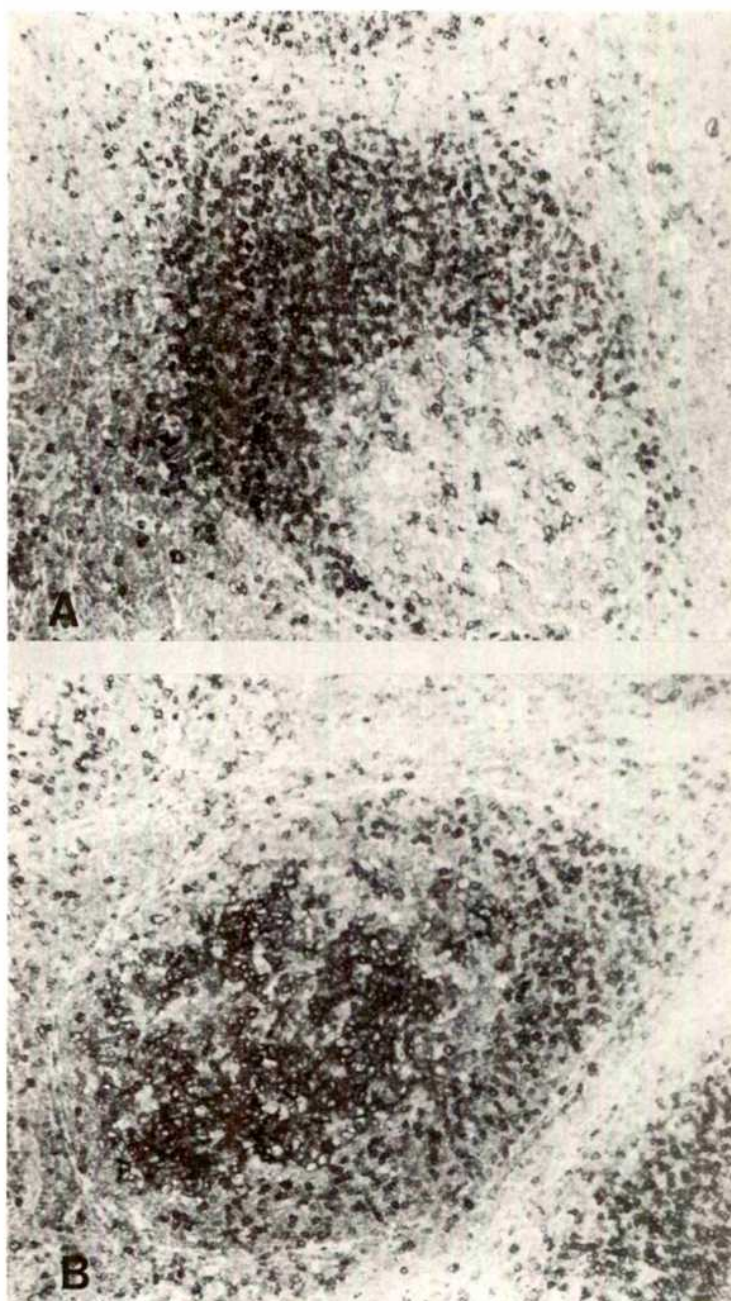


Fig 1. Paraffin section of formalin-fixed normal human lymph node (A) and tonsil (B) stained with Ki-B3. Approximately 60% of follicular mantle cells and a minority of germinal center cells are positive (A). In human tonsil the minority of mantle cells are stained, whereas the majority of germinal center cells shows Ki-B3 reactivity. 5 μ m paraffin section; immunoperoxidase staining; original magnification $\times 140$; current magnification $\times 108$.

Table 4. Reactivity of Different Carcinomas With Ki-B3, Epithelial Membrane (HMFG2), and Leukocyte Common Antibody

Localization of Carcinoma	n	Ki-B3	HMFG2	Leukocyte Common
Breast	14	0/14	14/14	0/14
Intestinal tract	10	0/10	9/10	0/10
Uterus	2	0/2	2/2	0/2
Esophagus	3	0/3	3/3	0/3
Uterus	3	0/3	3/3	0/3
Kidney	3	0/3	3/3	0/3
Skin	5	0/3	2/3	0/3
Urinary bladder	2	0/2	2/2	0/2
Prostate gland	1	0/1	1/1	0/1
Ovary	1	0/1	1/1	0/1
Lymphoepithelial Carcinoma	9	0/9	5/8	0/9
Others	2	0/2	2/2	0/2

The results with separated leukocyte fractions have been surveyed in Table 4. Whereas granulocytes were invariably negative, ~3% of monocytes showed a positive reaction. In cytopspins of peripheral T cells <21% of the mononuclear cells were positive. Preparation of B cells showed 91% positive lymphoid cells.

Double labeling experiments showed that 95% of cells in the B lymphocyte fraction expressed To15 and Ki-B3 and 2% of the cells in the T lymphocyte fraction expressed Leu4 and Ki-B3, simultaneously.

The staining results with normal tissue samples are specified in Table 1. All tissue types showed a negative reaction to Ki-B3, except for elastic fibers in the wall of blood vessels. In line with reactivity in tonsillar tissue, lymphoid cells were positive in B cell areas of lymph nodes and spleens including marginal zone cells. Starry sky macrophages in germinal centers of lymph nodes as well as alveolar and peritoneal macrophages were Ki-B3 negative. The results were identical on paraffin as well as on cryostat sections. Bone marrow cells were invariably negative except some single monocytes and lymphoid cells (Table 3).

The details of the 58 investigated carcinoma cases are given in Table 4. All carcinomas showed an invariably

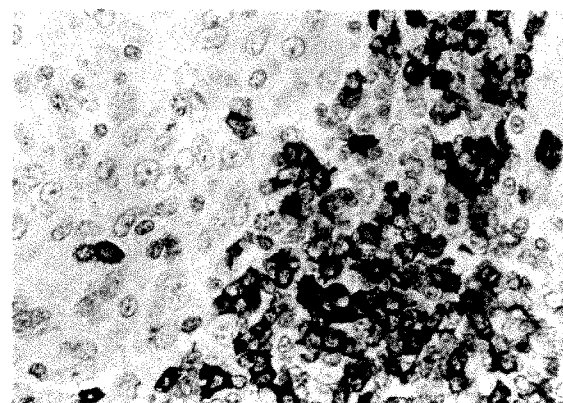


Fig 2. Paraffin section of formalin-fixed lymph node infiltrated by an undifferentiated carcinoma (left) stained with Ki-B3. Carcinoma cells are invariably negative. On the right side remnants of preexisting lymphatic tissue positive to Ki-B3. 5 μ m paraffin section; immunoperoxidase staining; original magnification $\times 560$; current magnification $\times 392$.

negative reaction (Fig 2). The same applied to the cases of 19 malignant melanomas. Forty-three of the 44 sarcomas showed a negative reaction. Only one case of an undifferentiated sarcoma was Ki-B3 positive and additionally revealed a positivity for the common leukocyte antibody. This tumor was negative for the epithelial membrane antibody and antibodies recognizing the intermediate filament desmin. The nine cases of nasopharyngeal lymphoepithelial carcinoma (SCHMINCKE tumor) and five cases of neuroendocrine carcinoma revealed a negative reaction for Ki-B3.

Of the 90 cases of low-grade B cell malignant lymphoma, all of which expressed the LC-antigen, 63 showed a clear-cut positivity in more than 90% of the tumor cells. Only nine cases were partly positive, whereas 18 were completely negative. The different lymphoma subtypes according to the Kiel-Classification¹⁵ and to the Working Formulation¹⁶ are detailed in Table 5.

Of the 87 high-grade B cell lymphomas, all of which expressed the LC-antigen, 70 were Ki-B3 positive in more than 90% of the tumor cells (Fig 3). Only one centroblastic

Table 5. Reactivity of Ki-B3 With Different Subtypes of Low-Grade Non-Hodgkin Lymphomas

Lymphoma Type	Working Formulation	n	Positive*	Partly Positive†	Negative
B-CLL	m.l., small lymphocytic	22	17	3	2
LP-IC	m.l., small lymphocytic	13	6	3	4
CB-CC	m.l., follicular	28	16	3	9
CC	m.l., diffuse, small cleaved	15	12	0	3
HCL		9	9	0	0
plasmacytoma		3	3	0	0
CB	m.l., diffuse, large cell	26	23	0	3
IB	m.l., diffuse, large cell, immunoblastic	11	7	0	4
LB-B	m.l., small, non-cleaved	9	8	0	1
Burkitt	m.l., small, non-cleaved	11	10	0	1
Multilobated		2	2	0	0
B unclassified		28	22	0	6

Abbreviations: B-CLL, chronic lymphocytic leukemia, B type; LP-IC, lymphoplasmacytoid immunocytoma; CC, centrocytic; HCL, hairy cell leukemia; CB, centroblastic; IB, immunoblastic; LB-B, lymphoblastic B.

*More than 80% of tumor cells positive.

†Between 40% and 80% of tumor cells positive.

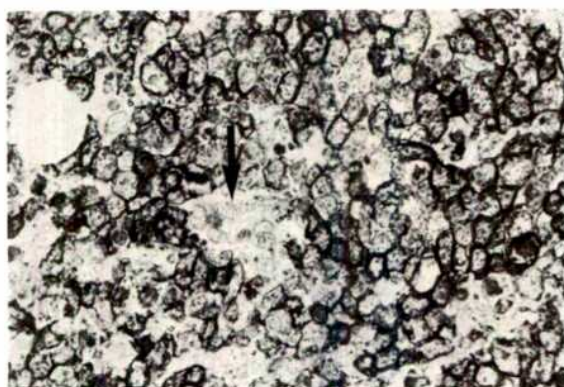


Fig 3. Paraffin section of formalin-fixed salivary gland infiltrated by a high-grade B cell lymphoma stained by Ki-B3. Note the negative reaction of a glandular duct (→). 5 μ m paraffin section; immunoperoxidase staining; original magnification $\times 560$; current magnification $\times 392$.

lymphoma showed a positivity in $\sim 15\%$ of the cells. Fifteen cases were completely negative. Detailed diagnosis and reactivity to Ki-B3 are given in Table 5.

All the cases positive for Ki-B3 on cryostat section were also positive on paraffin sections. The same was true for the negative cases. Exceptions were not observed.

Forty peripheral T cell lymphomas investigated showed a completely negative reaction in the tumor cells. Of 15 T lymphoblastic lymphomas, four cases showed a positive reaction to Ki-B3 (Table 6). Ten of 25 cases of myeloid or myelomonocytic leukemia showed a positive reaction with Ki-B3.

Ki-B3: Antigen characterization. After surface iodination of LICR-LON-HMY-2 cells, precleared lysate was precipitated with MoAb Ki-B3 and analyzed by SDS-PAGE. Under reducing conditions a heavily labeled band of ~ 220 kD was found (Fig 4). An identical electrophoretic mobility of the corresponding antigen was obtained when the glycoprotein fraction of cell lysate was used, indicating that the Ki-B3 antigen represents a 220 kD glycoprotein that is expressed on the cell membrane. For control MoAb, PA-1 recognizing the transferrin receptor (G. Moldenhauer, unpublished data) and an irrelevant MoAb of the same isotype as Ki-B3 (IgG1) were applied.

Table 6. Reactivity of Ki-B3 With Different Thymic and Postthymic T Cell Lymphomas

Lymphoma Type	Working Formulation	n	Ki-B3 Positive
T-LB		15	4
T-CLL	m.l., small lymphocytic	2	0
T zone	m.l., large cell, immunoblastic polymorphous	12	0
T-AILD		8	0
Lennert's Lymphoma	m.l., large cell, immunoblastic, epitheloid cell component	2	0
T-IB	m.l., large cell, immunoblastic	1	0

Abbreviations: T-LB, lymphoblastic T; T-CLL, chronic lymphocytic leukemia, T type; T-AILD, T cell lymphoma angio-immunoblastic type; T-IB, T immunoblastic.

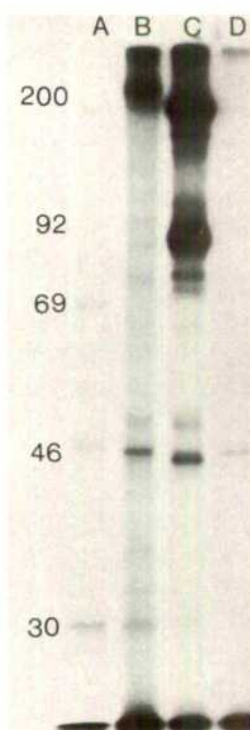


Fig 4. Radioimmunoprecipitation slab gel electrophoresis using ^{125} I-labeled LICR-LON-HMY2 cells under reducing conditions. Lane A, molecular weight standards; lane B, MoAb Ki-B3 precipitating a 220 kD molecule; lane C, MoAb Pa-1 detecting the transferrin receptor (gp 180/90 dimer); lane D, irrelevant MoAb of mouse IgG1 isotype.

DISCUSSION

Immunohistochemistry has been of considerable aid in the classification of tumors. The detection of cytoplasmic immunoglobulins in formalin-fixed and paraffin-embedded specimens of malignant lymphomas enabled the clear diagnosis of their B cell nature.³²⁻³⁶ The introduction of MoAbs allowed the visualization of a further large number of cellular antigens. The only disadvantage is that the majority of these antigens are easily destroyed or masked by routine fixation with formalin.³⁷ In this report we have introduced a new MoAb that recognizes the majority of normal and neoplastic B cells in formalin-fixed and paraffin-embedded tissues.

The reaction pattern of Ki-B3 in lymphoid tissue, recognizing $\sim 50\%$ to 80% of follicular mantle cells and $\sim 50\%$ to 60% of germinal center cells, is not identical to that of any other B cell antibody described so far. This finding is further underlined by the precipitation of a 220,000 D antigen, a molecular weight described so far only for common leukocyte antigen.^{2,10,38} It is now well established that the leukocyte common (LC) antigen is not a single entity but comprises a group of probably related molecules. Besides MoAbs recognizing the T200 antigen that is equally expressed on all lymphoid cells and monocytes/macrophages, there is another group of MoAbs precipitating a 220,000 D antigen and showing a similar but not identical reactivity pattern as LC antibodies.³⁹ They are reacting with B and T cells but not with granulocytes, and only a portion of 50% to 80% of bone marrow cells. Additionally this LC related antigen is found on some epithelial structures, such as tubules of human kidney.^{40,41} A somewhat more restricted reaction pattern concerning lymphatic cells is described for the MoAb F8-11-13.⁴²

Although Ki-B3 also precipitates a 220,000 D antigen

there are some striking differences in its reactivity pattern. Ki-B3 does not react with epithelial cells. Furthermore, the vast majority (if not all) peripheral T cells, as well as bone marrow cells, are negative. For this reason, Ki-B3 very likely recognizes a new subtype of the LC antigen that is predominantly expressed on B cells.

Other MoAbs, like LN1 and LN2, both recognizing B cell antigens in B5-fixed paraffin-embedded material are clearly different from Ki-B3. In contrast to Ki-B3, they neither react with formalin-fixed tissue samples nor show the same restricted reactivity, as they react with different epithelial cells (LN1) or with a small percentage of malignant melanomas (LN2).¹³

Even when using acetone-fixed cryostat section, we were not aware of any B cell-associated MoAbs that showed an identical reaction pattern to Ki-B3.^{8,43-52}

As Ki-B3 detects only a well-defined portion of lymphoid cells in the B cell areas of lymphoid tissue, it is not surprising that the majority (but not all) B cell neoplasms are stained. Interestingly, among those high-grade B cell lymphomas that are positive for Ki-B3, 60% did not express surface immunoglobulins on cryostat sections or cytoplasmic Ig in formalin-fixed material (details not provided in this report). Under routine conditions such tumors would not have been diagnosed as B cell lymphomas.

Of the 90 cases of low-grade B cell lymphomas, 72 were completely, or in part, positive to this antibody. The positive cases included all cases of hairy cell leukemia investigated in this study and three cases of plasmacytoma. Other B cell antibodies with a broad B cell reactivity, such as CD19 or CD22 antibodies, are not able to detect plasma cells or plasmacytomas even on cryostat sections. Since all cases of peripheral T cell lymphomas were invariably negative on

cryostat as well as on paraffin sections, Ki-B3 proves to be a reliable reagent in detecting and differentiating the vast majority of B cell lymphomas.

The T lymphocyte-derived lymphomas were considered the only exception, as was demonstrated by the unclear staining of four of 15 T lymphoblastic lymphomas. Normal thymus gland does not react with Ki-B3. Assuming that T lymphoblastic lymphomas and leukemias originate from thymic cortex cells and immature bone marrow T cells, the positive cases probably correspond to the differentiation stage of immature bone marrow or prethymic T cells. The same may be true for those myeloid or myelomonocytic leukemias reacting with Ki-B3 since normal immature cells of granulopoiesis (promyelocytes or myelocytes) are not stained by Ki-B3.

Besides the thymus gland, all other human tissues showed a negative reaction as did all carcinomas and sarcomas. For this reason Ki-B3 also allows a reliable differentiation between large-cell lymphomas of B type and carcinomas. This is an important observation as it is known that epithelial membrane antigens are also detectable on some high-grade lymphomas and for this reason do not allow a clear cut distinction of lymphomas and carcinomas.⁵³ The same is true for antibodies staining intermediate filaments, such as desmin and vimentin, that can be detected in mesenchymal cells as well as carcinomas.⁵⁴

The results clearly demonstrate that the MoAb Ki-B3 detects a new formalin resistant subtype of the LC antigen that is predominantly expressed on B cells. Therefore, this antibody is useful in routine hematology especially for the detection and differentiation of high-grade B cell lymphomas.

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T Cell Rearranging Gene γ : Diversity and mRNA Expression in Fresh Cells From T Cell Acute Lymphoblastic Leukemia

By Denis Le Paslier, Zhu Chen, Pascale Loiseau, Daniel Cohen, and François Sigaux

Rearrangement and in most cases expression of the T cell rearranging genes γ (TRG γ) and T cell antigen receptor β chain (TCR β) genes were studied in 19 cases of T cell acute malignancies where the surface phenotype is representative of the different stages of thymic maturation. TCR α gene transcription was also studied. TRG γ and TCR β genes were found to be rearranged in all but one case. The TRG γ rearrangement pattern seen in most cases is compatible with biallelic rearrangement by loop excision involving the J γ 2 regions. The sizes of all but two rearranged bands were identical to those of the rearranged bands seen in polyclonal T lymphocytes also studied in this work. One identical-sized band was found in 11 of the 18 rearranged

cases. The expression of TRG γ mRNA (transcripts of 1.6 kilobases [kb]) was highly variable from case to case and did not correlate with the stage of differentiation of the malignant cells, the expression of the molecules CD4 and CD8, the expression and size of the transcripts of the TCR β genes, and the transcription of TCR α genes. In one CD3+ case, strong expression of the TRG γ transcripts coexisted with the exclusive presence of TCR β mRNA of 1.0 kb. The cells from this case did not react with anti-Ti antibody and exhibited no natural killer activity. These findings are suggestive of a malignancy that may express the recently isolated CD3-TRG γ complex.

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A WIDE BODY OF EVIDENCE supports the view that the classic T cell antigen receptor (TCR) is a complex comprised of the multichain molecule CD3 (T3) and the two molecules T α and T β whose genes undergo somatic rearrangements during thymic ontogeny of T cells.¹⁻³ Transfection experiments have established that, at least in some T cells, the dimer T $\alpha\beta$ determines both antigen recognition and its restriction by the major histocompatibility complex (MHC), which is characteristic of T cells.⁴ During attempts to isolate the genes encoding the T α chain, other genes that also undergo somatic rearrangements in T cells were brought to light in mice⁵⁻⁷ and then in humans.^{8,9} The structure of these genes, termed T cell rearranging genes γ (TRG γ) or T cell γ chain genes, has recently been elucidated at least in part for humans. Nine¹⁰ to 12¹¹ variable segments (V γ), including at least five pseudogenes, are located upstream of two strongly homologous regions, each comprising a junctional segment (J γ) and a constant region (C γ).^{8,12} The V γ segments can recombine with both regions J γ 1 and J γ 2 and also with a segment called J γ P by Lefranc et al¹⁰ that is located at about 4 kilobases (kb) in 5' of the J γ 1 region (Fig 1). The diversity generated by TRG γ is basically of junctional origin (including N diversity). There is no evidence of D γ segments.^{10,13} We and others¹⁴⁻¹⁶ have shown that the rearrangement of TRG γ is not restricted to normal and neoplastic T cells. In a recent work,¹⁴ we have shown that TRG γ are frequently rearranged but not transcribed in the malignant cells of B lineage acute lymphoblastic leukemia (ALL). Although the structure of TRG γ has been elucidated to a large extent, the nature and function of the presumed products of TRG γ remain a mystery.¹⁷ In mouse thymocytes,^{18,19} TRG γ rearrangement and mRNA expression precede those of the genes TCR β and TCR α , which suggests that TRG γ may play a crucial role in early thymic ontogeny of T cells. As shown by recent research using cross-linking experiments and immunoprecipitation by anti-CD3 monoclonal antibodies (MoAbs) and by antisera directed against synthetic V γ and C γ peptides, the product of TRG γ could be a 55-kiloDalton (kD) protein associated with the CD3 molecule.²⁰ One of the more attractive hypotheses is that this TRG γ -CD3 complex could be used by certain T cells (CD3-positive, Ti-negative) as an antigen receptor.²⁰

T cell acute lymphoblastic leukemias (T-ALL) can be

considered as clonal proliferations of cells arrested at more or less early stages of thymic differentiation and are models of considerable interest.²¹ The present study investigated the rearrangement and expression of mRNA of TRG γ in fresh cells from a large series of T-ALL and one patient with T blast crisis of chronic myeloid leukemia (CML). The surface phenotype of malignant cells was determined by using a panel of MoAbs directed against associated or restricted T antigens by which the stage of differentiation of the malignant cells could be determined.^{21,22} Rearrangement of TCR β genes and transcription of TCR β and TCR α genes were also studied in most cases. We showed that the TRG γ rearrangement seen in all but one case most often involves the J γ 2 region of both alleles and probably uses the same V γ segments as those used by mature polyclonal T cells. It was also found that TRG γ mRNA expression, variable from one case to another, does not correlate with surface phenotype, transcription of TCR α and TCR β genes, and stage of differentiation of the malignant cells. Finally, the intense expression of TRG γ mRNA in one CD3-positive, Ti-negative case expressing only 1.0-kb TCR β mRNA is suggestive of a malignancy that may express the recently isolated CD3-TRG γ complex.^{20,23}

MATERIALS AND METHODS

Cells

Malignant cells from the blood or bone marrow of 18 T-ALL patients and one patient with T lymphoblastic blast crisis of CML

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were separated on a Ficoll gradient and then used as is or after conservation in liquid nitrogen. Morphological and immunologic characterization was based on the usual criteria.^{22,24,25} The surface phenotype of the malignant cells was determined by a standard method of indirect immunofluorescence and cytofluorograph reading (Ortho 50 H; Ortho Diagnostics, Westwood, MA) using a large panel of MoAbs that recognize T-associated or T-restricted antigens (anti-CD1 to -CD5, -CD7, and -CD8) and B-restricted antigens (CD19 to CD22). A number of other antibodies were also used (anti-CD10, OKT9, OKT10, My7, MY9, OKM1, OKM5, anti-platelet glycoprotein Ib and IIb/IIIa, and anti-MHC class II molecules DR, DP, and DQ).²⁶ The specificity of these antibodies has been widely published. The WT31 antibody interacting with nonpolymorphic determinant expressed on Ti was also used in some experiments. A cell population was considered positive if more than 30% of the neoplastic cells reacted with the MoAb. Table 1 summarizes the surface phenotypes of the cells used. B-restricted antigens were not detected in any of the cases, nor were myeloid antigens (anti-MY9), whereas all the cases tested expressed the pan-T molecule CD7.²⁷ The CD3 molecule was expressed in five of 18 cases of T-ALL, four of which also expressed either CD4 or CD8 (stage III of Reinherz et al²¹). Of the CD3-negative cases, six expressed at least one of the common thymocyte antigens (stage II) and seven only pan-T markers (CD5 and CD7) (stage I or I/II). OKM1 antigen was expressed in case 8. The cells of the T cell blast crisis of CML were of a typical common cortical phenotype. Among the cases analyzed in this paper, three are described elsewhere.²⁸⁻³⁰

To compare the patterns of rearrangement of TRG γ in T cell malignancies and in polyclonal T cells, T cells from healthy donors were studied after E rosetting. Granulocytes, monocytes, polyclonal B cells transformed by Epstein-Barr virus (EBV) or not, blasts of

acute myeloid leukemia patients and the CEM line were also used in some experiments.

Southern Blots

Genomic DNA was extracted according to standard methods. DNA samples were digested by the restriction endonucleases *Bam*HI, *Hind*III, and *Eco*RI under conditions recommended by the manufacturer (Promega Biotec, Madison WI). The restriction fragments were separated in 0.6% agarose gel by electrophoresis at 40 V for 24 hours and transferred onto a hybridization membrane (Hybond-N from Amersham Corp, Arlington Heights, IL). The prehybridization and hybridization were performed at 65°C in 6 $\times$ SSC (1 $\times$ SSC is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate, pH7), 0.2% sodium dodecyl sulfate (SDS), 10% (wt/vol) dextran sulfate, and sonicated denatured salmon sperm DNA at 200 μ g/mL. The filters were washed for 15 minutes in 2 $\times$ SSC and 0.2% SDS at room temperature, 15 minutes in the same solution at 65°C, 30 minutes in 0.2 $\times$ SSC and 0.2% SDS, and then 30 minutes in 0.1 $\times$ SSC and 0.2% SDS at 65°C. The blots were autoradiographed for two to four days.

Northern Blots

The extraction of total mRNA was performed as described³¹ by using the guanidium-cesium chloride method. The cell pellet was lysed by vortexing into 6 mol/L guanidium isothiocyanate, 5 mmol/L sodium citrate, 0.1 mol/L β -mercaptoethanol, and 0.5% sarkosyl. The lysate was then centrifuged over a gradient of 5.7 mol/L cesium chloride at 77,000 g for 16 hours at 20°C. The RNA was precipitated in 70% ethanol and resuspended in diethylpyrocarbonate-treated water. Fifteen micrograms of total RNA were electrophore-

Table 1. Immunophenotype, Rearrangement, and Expression of TRG γ and TCR Genes

No.	Surface Phenotype										TRG γ				TCR β			TCR α		
	CD7	CD5	CD2	T10	T9	CD1	CD4	CD8	CD3	CD10	Rearrangement				Rear- range- ment	mRNA		mRNA		
											Type*	H	E	mRNA		1.0 kb	1.3 kb	1.3 kb	~1.6 kb	
1	+	-	-	-	-	-	-	-	-	+/-	R	4	e, f	c, h	++	R	-	-	-	-
2	+	+	-	+	+	-	-	-	-	-	R	+	c	e	++	R	+	+/-	-	-
3	+	+	-	+	+	-	-	-	-	-	R	4	f, g	g, c	+	R	+/-	-	-	-
4	+	-	-	+	+	-	+/-	-	-	-	R	\$	a, c	a, e	ND	R	ND	ND	ND	ND
5	+	+	+/-	+	+	-	-	-	-	-	R	J	-	-	ND	R	ND	ND	ND	ND
6	+	+	+	+	-	-	-	-	+/-	-	R	r	c	e	+/-	R	-	++	-	++
7	+	+	+	+	+	-	+/-	-	-	+/-	R	4	a, f	a, c	+/-	R	+/-	-	-	-
8†	+	+	+	+	+	-	-	-	-	-	G	-	-	-	ND	G	ND	ND	ND	ND
9	+	+	-	+	+	+	+	-	-	+	R	°	b	f	++	R	-	+/-	-	-
10	+	+	+	+	+	-	+	-	-	+/-	R	4	c, d	d, e	+++	R	-	-	-	-
11‡	+/-	+	+	-	-	+	+	+	-	-	R	4	c, e	b, e	-	R	-	-	-	-
12	ND	+	+	+	-	+	+	+	-	-	R	+	c	e	+	R	+/-	+	-	-
13	+	+	+	+	+	+/-	+	+	-	-	R	4	b, c	e, f	+++	R	-	++	+/-	-
14	+	+	+	-	-	-	+	+	-	-	R	4	b, c	e, f	+/-	R	-	++	++	-
15	+	+	+	-	+	-	-	+	+	-	R	4	e, f	c, h	-	R	-	+++	-	++
16	+	+	+	+	+	-	-	+	+	-	R	+	c	e	ND	R	ND	ND	ND	ND
17	+	+	+	+	-	+/-	-	-	+	-	R	°	f	c	+++	R	++	-	-	+
18	+	+	+	-	-	-	+	+	+	-	R	\$	a, c	a, e	-	R	-	+++	+	+/-
19	+	+	+/-	+	+	+/-	+/-	-	+	-	R	4	e, h	c, h	ND	R	ND	ND	ND	ND

Abbreviations: ND, not done; H and E, rearranged bands in DNA digested by *Hind*III and *Eco*RI, respectively (for details see the text); +/-, 20% to 30% of cells positive for the surface marker (for immunophenotype) or very weakly positive (for mRNA expression).

*4, biallelic J γ 2 rearrangement; +, two rearranged bands with *Bam*HI but only one with *Hind*III and *Eco*RI; \$, two rearranged bands with *Hind*III and *Eco*RI but only one rearranged band with *Bam*HI; J, monoallelic rearrangement involving a J segment localized 5' to the *Hind*III and *Eco*RI sites preceding the J γ 2 region but included in the *Bam*HI fragment containing J γ 2 (see Fig 1); r, rearrangement not interpretable by the loop excision mechanism; °, deletion of the J γ 1 = J γ 2 region on one allele and J γ 2 rearrangement on the other.

†Seventy-four percent of malignant cells from this case are OKM1-positive.

‡T cell acute transformation of CML.

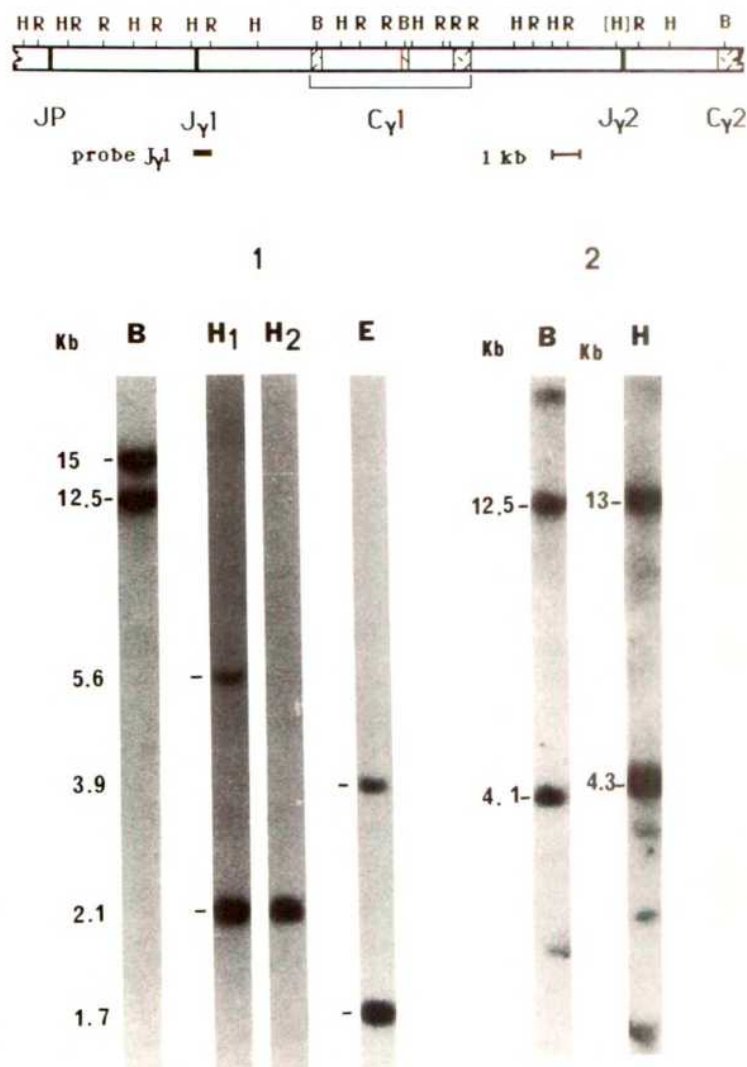


Fig 1. (Top) Organization of the J γ and C γ regions. This restriction map is adapted from Lefranc et al and Quertermous.^{8,10,11} The relevant HindIII (H), EcoRI (E) and BamHI (B) sites are indicated; not all restriction enzyme sites are shown. JP is the joining region defined in ref. 10. The polymorphic HindIII site is indicated in parentheses. (Bottom) Germ line configuration of J γ (1) and C γ (2) segments in DNA digested by BamHI (B) and EcoRI (E). H1 and H2 show the polymorphism of the HindIII site 5' upstream to the J γ 2 segment.

sed through a 1.2% agarose-formaldehyde gel and transferred to nitrocellulose or nylon membrane. The prehybridization and hybridization were performed in $4 \times$ SSPE ($1 \times$ SSPE is 0.18 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L NaH_2PO_4), 0.2% SDS, 0.1% Nappi, and 5 $\mu\text{g}/\text{mL}$ of heparin. The filters were washed for one hour in $1 \times$ SSPE 0.1% SDS and $0.5 \times$ SSPE 0.1% SDS at 68°C.

DNA Probes

The TCR β probe is a cDNA fragment specific for the constant region of the β chain of the TCR (a BglII/BglII fragment of the 4D1 cDNA³²) that was provided by J.L. Strominger. The TCR α probe is the pGA5 cDNA provided by E. Palmer.³³ The J γ probe is a subcloned EcoRI-HindIII fragment of the MH60 J γ clone that was provided by T.H. Rabbits⁸ (Fig 1). The C γ probe is the 0.7-kb BamHI fragment (part of exon 2 and all of exon 3) of the PHT γ 9 cDNA encoding for a VJC gene that was provided by J.L. Strominger.¹¹ The entire PHT γ 9 cDNA probe was used to detect the TRG γ transcripts. Probes were ³²P-labeled by nick translation or by oligo-labeling (multiprime labeling kit from Amersham).

Interpretation of the Southern Blots

J γ . Figure 1 shows the bands seen in germ line DNA digested by the restriction enzymes BamHI, HindIII, and EcoRI. No polymor-

phism was observed for the BamHI sites in the control samples (granulocytes, monocytes, and B lymphocytes from four healthy donors; blasts from nine patients with typical acute myeloid leukemia; and polyclonal B cell lines obtained after transformation of the blood of 20 healthy donors by EBV). As previously described,⁸ polymorphism was noted for the HindIII site situated upstream to the J γ 2 segment, which resulted in a 2.1-kb band in some individuals and both 2.1- and 5.6-kb bands in others (Fig 1). Southern blots were interpreted by assuming a loop excision mechanism without reintegration and using the following nomenclature¹⁴: type 1, monoallelic J γ 1 rearrangement; type 2, biallelic J γ 1 rearrangement; type 3, monoallelic J γ 2 rearrangement; type 4, biallelic J γ 2 rearrangement; and type 5, J γ 1 rearrangement on one allele and J γ 2 on the other.

C γ . When germ line DNA is hybridized with the C γ probe (corresponding to a part of exon 2 and all of exon 3), two BamHI bands of 12.5 and 4.1 kb (corresponding respectively to C γ 1 and C γ 2) and two HindIII bands of 13 and 4.3 kb (C γ 2 and C γ 1, respectively) can be seen (Fig 1).¹⁴ Monoallelic or biallelic J γ 1 rearrangements cannot be detected with this probe. On the other hand, J γ 2 rearrangement results in reduced intensity (monoallelic rearrangement) or disappearance (biallelic rearrangement) of the 12.5-kb BamHI band and the 4.3-kb HindIII band.

TCR β . TCR β gene rearrangements were analyzed as previously described.³⁴

RESULTS

TRG γ Rearrangement in Polyclonal T Cells and T Cell Acute Malignancies

TRG γ rearrangement was first studied in polyclonal T lymphocytes after E rosetting. A representative result obtained after hybridization with the J γ probe is shown in Fig 2. Six faint rearranged bands (termed Ha to Hf and Ea to Ef) were seen in DNA digested by *Hind*III and *Eco*RI, respectively, in addition to the germ line bands observed in the granulocytes, monocytes, and B lymphocytes of the same individual. Because the hybridization of the same filter with a TCR β probe shows a pattern characteristic of polyclonal T cells (almost total disappearance of the 12-kb *Eco*RI band),

the presence of germ line bands in the DNA hybridized with the J γ and C γ probes seems not to be due to a contamination of the E fraction (for example, by natural killer [NK] E+ cells³⁵) but is suggestive of the absence of rearrangement in some polyclonal T cells or of monoallelic rearrangement in at least a fraction of them. The CEM line, which served as a control in this experiment, exhibited type 4 rearrangement (biallelic J γ 2) with two bands of the same size of the Hc-Ee and He-Eb bands found in polyclonal T cells.

Of the 19 cases of T cell acute malignancies studied, only one instance of germ line pattern was found (case no. 8) (Table 1). In nine of 18 cases of rearrangement, the pattern observed can be explained by biallelic rearrangement of region J γ 2 (type 4). In two cases (nos. 4 and 18), the pattern

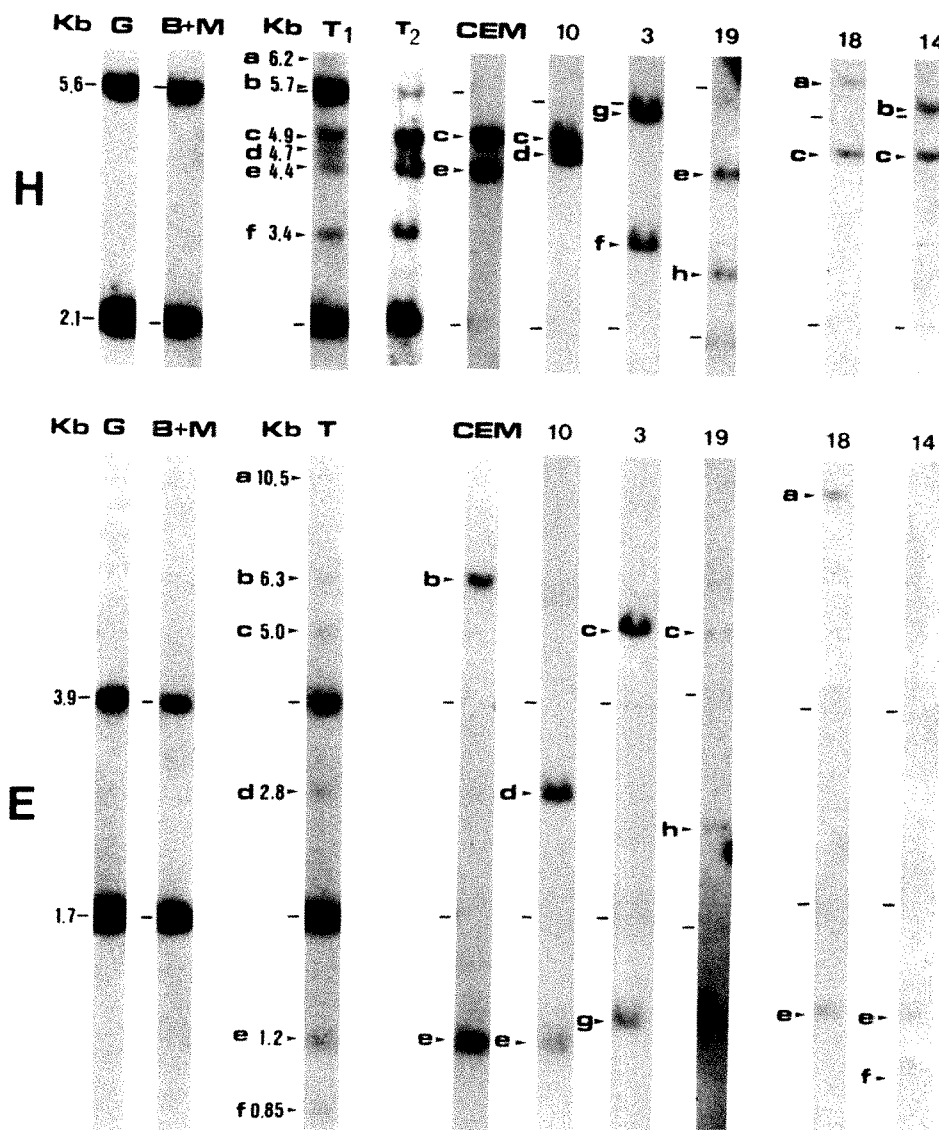


Fig 2. The germ line and rearranged bands in DNA digested by *Hind*III (H) and *Eco*RI (E) and hybridized with the J γ 1 probe. G, normal granulocytes; B + M, normal peripheral blood B lymphocytes and monocytes; T1 and T2, normal peripheral polyclonal T cells (E+ fraction). The rearranged band Hb is more easily seen in subjects without the polymorphic 5.6-kb *Hind*III band (T2). CEM, T-ALL cell line. The others are the samples from the T-ALL patients, their case number located above each lane. —, germ line bands; >, rearranged bands. A correspondence between the different rearranged bands in DNA digested by *Hind*III and *Eco*RI has been established as follows: Ha-Ea, Hb-Ef, Hc-Ee, Hd-Ed, He-Eb, Hf-Ec, Hg-Eg, Hh-Ec, He-Eh.

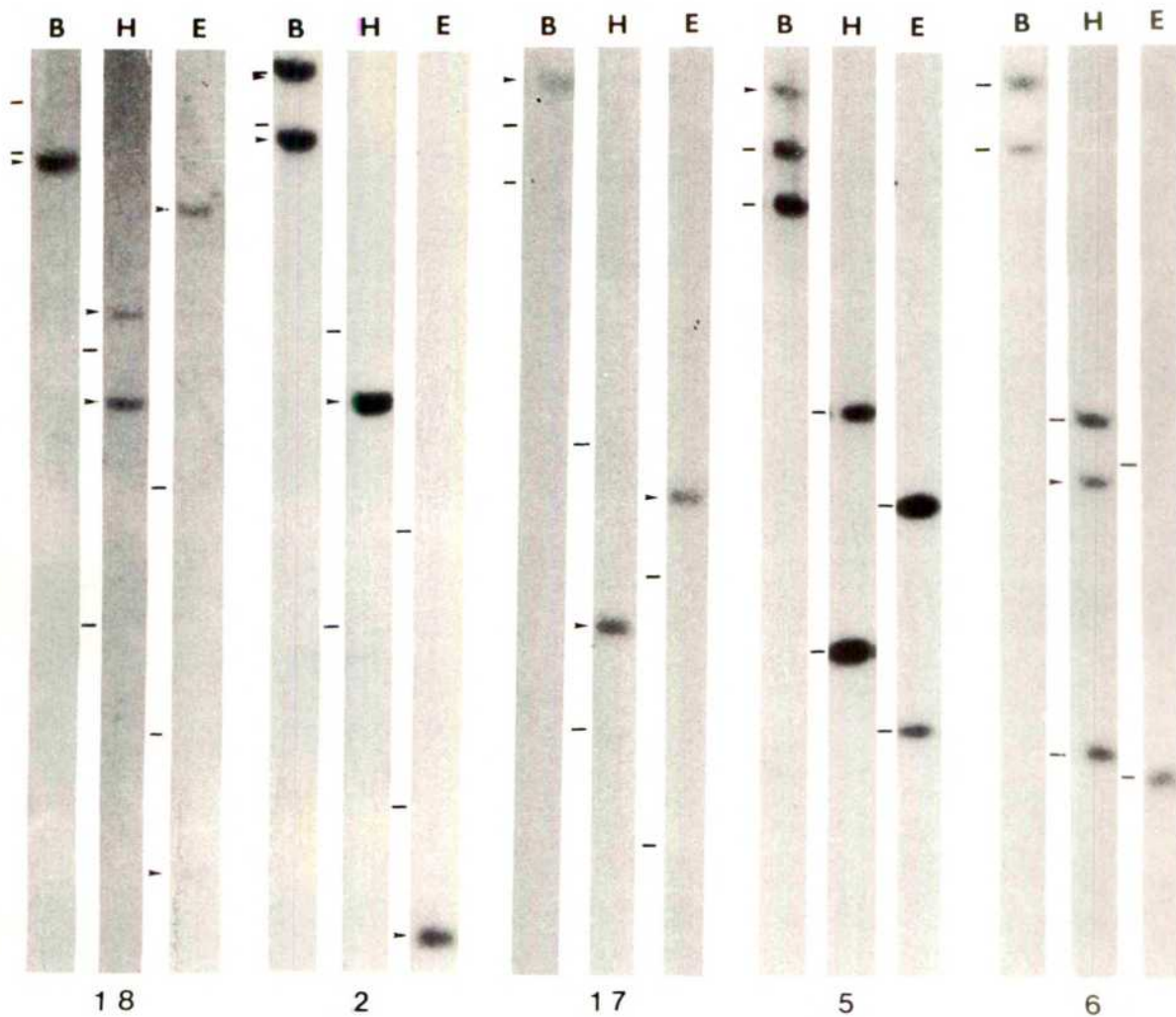


Fig 3. Atypical patterns of J γ rearrangement observed in T-ALL. DNAs are digested by *Bam*HI (B), *Hind*III (H) and *Eco*RI (E). Case numbers are listed under each triplet of lanes (see Table 1). —, germ line bands; >, rearranged bands.

obtained with the DNA digested by *Hind*III and *Eco*RI was also consistent with type 4 rearrangement, but a single rearranged band was seen in DNA digested by *Bam*HI. In case nos. 2, 12, and 16, two rearranged bands were seen in *Bam*HI digests of DNA but only one for the enzymes *Hind*III and *Eco*RI (Fig 3). Since the restriction patterns of certain V γ regions were strongly homologous,¹⁰ these results might conceivably result from comigration of certain fragments, even though they correspond to type 4 rearrangement. In case nos. 9 and 17, the presence of a single rearranged band along with the disappearance of the germ line bands is consistent with J γ 2 rearrangement associated with the deletion of a fragment including at least the whole of the J γ 1-J γ 2 region on the other allele or possibly with a type 4 rearrangement using the same V γ segment on both alleles (Fig 3). In case no. 5 (characterized by one rearranged band with *Bam*HI and a germ line pattern with *Hind*III and *Eco*RI (Fig 3)), there seems to have been a monoallelic rearrangement of a joining region like the JP segment described by Lefranc et al.¹⁰ Finally, the type of rearrangement seen in case no. 6 remains uninterpretable on the basis of a loop

excision hypothesis (Fig 3). Rearrangement patterns for the different patients are summarized in Table 1.

All the rearranged bands obtained in polyclonal T cells after DNA digestion by *Hind*III and *Eco*RI were observed in T cell acute malignancies (Fig 2). Band Hc-Ee occurred in ten of 18 cases. In polyclonal T cells, these are the bands of highest intensity (Fig 3). In certain cases of T-ALL (nos. 1, 3, 15, and 19), there were additional bands—*Hind*III of 5.3 and 2.6 kb (Hg and Hh, respectively) and *Eco*RI of 1.3 and 2.1 kb (Eg and Eh, respectively)—that were not seen in the samples of polyclonal T cells studied. The number of cases of T-ALL was sufficient to establish a correspondence between the different rearranged bands observed after digestion by the two enzymes (Fig 2, see the legend). There was no correlation between surface phenotype and the presence of any given band.

TCR β Gene Rearrangement

TCR β genes undergo very early rearrangement—before the TCR α genes—during thymic ontogeny.³⁴ To establish whether a hierarchy by differentiation stage could be estab-

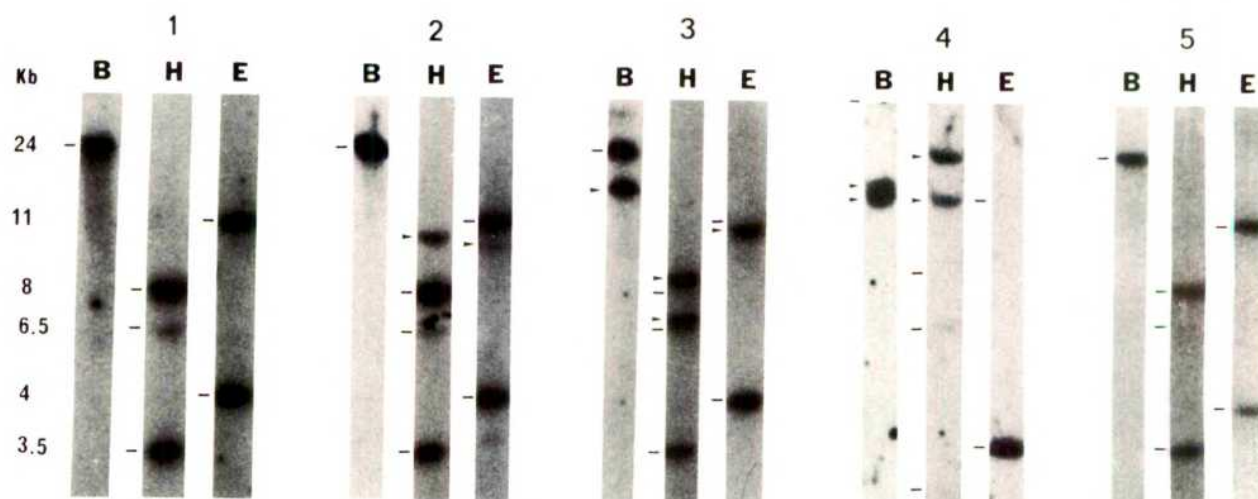


Fig 4. Germ line configuration and rearrangement of TCR β genes. (1) DNA from normal granulocytes digested by *Bam*HI (B), *Hind*III (H) and *Eco*RI (E). Several representative cases of T-ALL with differently rearranged TCR β gene patterns are shown: 2, case no. 5; 3, case no. 7; 4, case no. 9. The last triplet of lanes shows the only case (patient no. 8) whose TCR β genes remain unarranged.

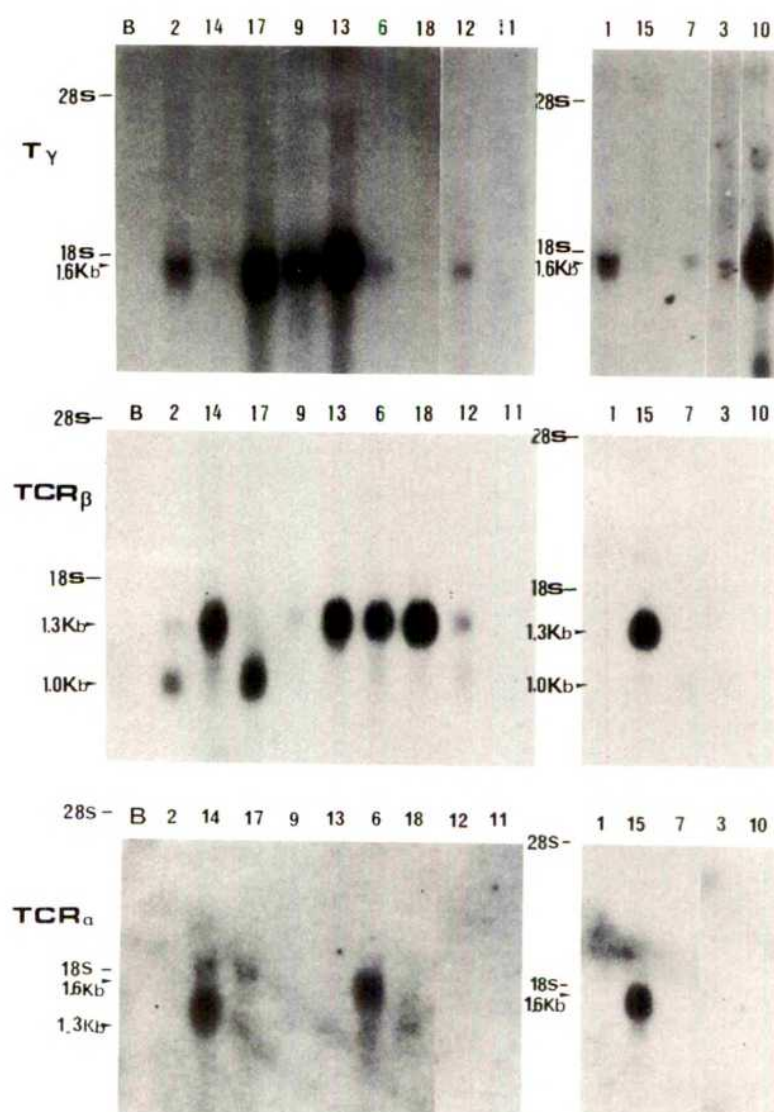


Fig 5. mRNA expression of the TCR γ and TCR β and TCR α genes. B, mixture of EBV-transformed polyclonal B cells lines taken as negative control. The other lanes are samples from the T-ALL patients, their case numbers located above each lane. The size of the mRNA is established with reference to the positions of 28S and 18S ribosomal RNA.

lished with respect to rearrangement of TCR β genes and TRG γ , the DNA previously hybridized with the TRG γ probes was dehybridized and rehybridized with the TCR β probe (which recognizes constant regions). All of the T-ALLs showing TRG γ rearrangement also exhibited TCR β gene rearrangement. Representative experiments are shown in Fig 4. In seven of the 17 analyzable cases, the observed pattern was consistent with biallelic J β 2 rearrangement by loop excision. In the sole case (case no. 8) where the TRG γ were not rearranged, a germ line pattern was also found for the TCR β genes (Table 1).

Expression of mRNA of TRG γ , TCR α , and TCR β Genes

Since TRG γ mRNA expression seems related to the stage of differentiation,^{18,19} it seemed interesting to study TRG γ mRNA expression in different cases of T-ALL. In 11 of the 14 cases studied a 1.6-(kb) transcript was found (Fig 5). The expression of TRG γ mRNA seems to show considerable quantitative variation and is not connected with the surface phenotype (Table 1), nor is it correlated with the expression and size of TCR β gene transcripts. In case nos. 3, 7, and 17, only 1.0-kb TCR β transcripts were found, which likely correspond to incomplete DJ rearrangements. Complete transcripts of 1.3 kb (potentially functional) were found at all stages of maturation, with maximum expression in CD3+ case nos. 15 and 18. TCR α gene transcripts were observed in six cases. TCR α mRNA of about 1.6 kb tend to be observed more frequently among the CD3-positive cases (Fig 5 and Table 1). Case no. 17 was exceptional in that only 1.0-kb TCR β transcripts were detected, whereas the CD3 molecule was clearly detected at the cell membrane. The cells did not react with the anti-Ti WT31 antibody (Fig 6). It is also

interesting to note that TRG γ expression was intense (Fig 5) and that the cells exhibit no NK activity (data not shown).

DISCUSSION

Our work has demonstrated the existence of a rearrangement of the TRG γ and TCR β genes in 18 of 19 cases of a series of acute T lymphoblastic malignancies whose surface phenotype illustrates the different stages of thymic maturation.²¹ In a single case (no. 8), with a common cortical phenotype, the TRG γ and TCR β genes were seen in germ line configuration. These findings along with those obtained with cases recently reported in the literature^{8,11,16} and those obtained in cell lines^{16,34} show that these genes are rearranged in the vast majority of ALLs of T phenotype. The lineage involved in patient no. 8 remains unclear. In this case, the malignant cells were clearly peroxidase-negative and expressed the OKM1 antigen but not myeloid antigens. Recently, it was shown that CD3-, NK cells (frequently OKM1-positive) do not rearrange TRG γ ³⁵ or TCR β genes.³⁶ Indeed, we have recently found TRG γ and TCR β genes in germinal configuration in CD3-, NK cells from a patient with a chronic expansion of large granular cells.³⁷ It is possible that the cells from case no. 8 reported in the present paper represent the neoplastic counterpart of NK precursors.

One of the objectives of this work was to discover whether there is any hierarchy in the rearrangement of the TRG γ and TCR β genes as there is in the case of TCR β and TCR α genes.³⁴ Our results do not support this hypothesis. It is nevertheless possible that our T-ALL cases only imperfectly illustrate the stages of thymic maturation³⁸ or that a hierarchy might be observed in more immature cells (prethymic?).

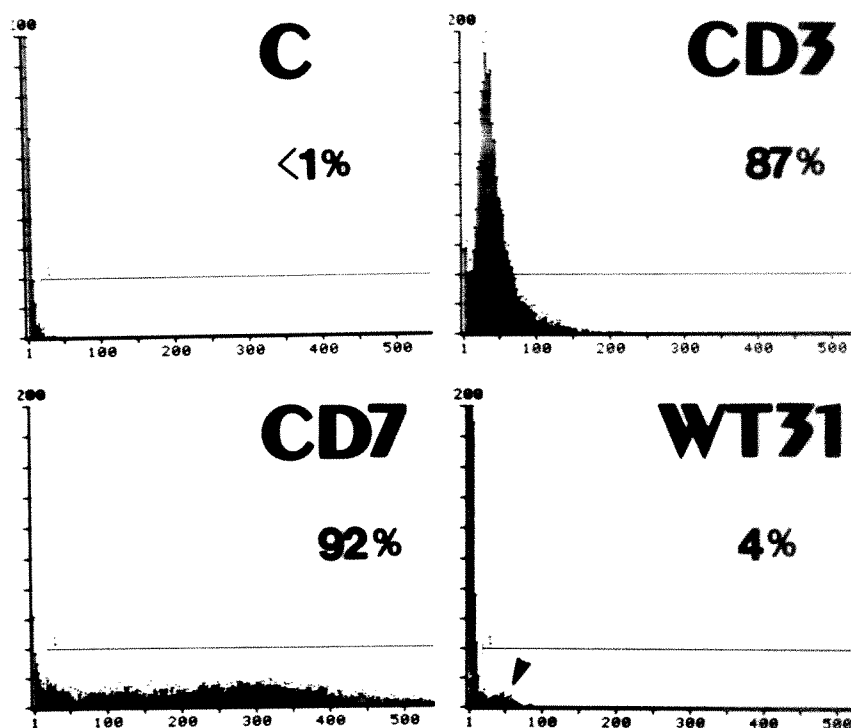


Fig 6. Partial surface phenotype of leukemic cells from case no. 17 as determined by indirect immunofluorescence. In the control experiment, cells were incubated with irrelevant antibody (C). Almost all cells were strongly labeled by anti-CD7 antibody. Eighty-three percent of the cells were positive for CD3, but only 4% carried the Ti molecule as detected by WT31. This WT31+ population (▴) served as the internal control of the test and may represent the persisting mature T lymphocytes in the blood (mixture of CD4+, CD8+ small scatter cells).

The demonstration of TRG γ rearrangement in most B lineage ALLs¹⁴ with TCR β gene rearrangement being found in only 30% to 40% of cases^{14,39} is evidence in favor of this hypothesis.

One interesting finding of the present study was that TRG γ rearrangement in T-ALL very often involves the J γ 2 segment on both alleles. On the other hand, the involvement of the J β 2 region in TCR β gene rearrangement was observed in only six of 17 cases interpretable in the hypothesis of loop excision. These findings are comparable with what can be deduced from the data of Tawa et al³⁹ (Table 1) and with Sangster and colleagues' data based on cell lines.³⁴ If a mechanism of allelic exclusion^{40,41} exists for TRG γ as for the other immunoglobulinlike genes, a good many of the TRG γ rearrangements must be nonfunctional in T-ALL. A large number of nonfunctional TRG γ transcripts have been demonstrated in mouse malignant T cell lines.^{42,43} In one series of human cell lines, only three of the nine rearranged fragments were found to be potentially functional by sequence analysis.¹⁰ The high rate of nonproductive rearrangements may result at least in part from N region diversity.¹³ It is interesting to note that in polyclonal T lymphocytes, as in human thymocytes,¹⁰ there are T cells in which TRG γ rearrangement is lacking or monoallelic. Thus this finding contrasts with what is observed in T-ALL where both alleles are usually rearranged. As already mentioned earlier, it may be that T-ALLs represent malignancies that develop preferentially from certain thymic cells.

Finding rearranged bands in the DNA of polyclonal T cells after hybridization with a J γ probe is an observation that is consistent with the small number of V γ segments (nine to 12)^{10,11} and the strong homology of the J γ 1 and J γ 2 regions.¹² The presence of merely six bands when nine to 12 could have been expected may be accounted for by comigration of certain fragments or by the preferential use of certain V γ segments. These findings contrast with those for the TCR β genes⁴⁴ (no rearranged bands are seen in polyclonal T cells) and are analogous to those recently obtained for TRG γ in human thymocytes by Lefranc et al.¹⁰ Using specific probes for certain V γ segments, these authors also assigned the V γ 9 fragment to a band corresponding to band Hd, the V γ 3 fragment to band He, and the segment V γ 4 to band Hc. When the patterns obtained in polyclonal T cells are compared with the patterns in T-ALL, identical-sized bands are found in the vast majority of cases. Nevertheless, two additional rearranged bands were seen only in T-ALL, thereby suggesting that certain V γ segments are little or not used in polyclonal T cells. It is also worth noting that, as in the mouse,⁶ certain V γ segments may be more often used than others. For instance, the Hc-Ee band is found in ten of 18 cases of rearranged T-ALL. These findings are analogous to those obtained with polyclonal T cells (Fig 3) and thymocytes¹⁰ where the intensity of these bands is higher than for the others. Finally, the pattern observed in some ALL cells upon *Eco*RI and *Hind*III digestion is not consistent with previously published maps¹⁰ and may be due to atypical rearrangement or involvement of previously unreported J γ or V γ segments. Cloning and further analysis of these rearranged fragments are in progress in our laboratory.

As shown in our study, expression of TRG γ mRNA by fresh cells from T-ALL is highly variable from case to case and is not correlated with CD4 and CD8 antigen expression. It should be underlined that the correlation between TRG γ mRNA expression and both function and MHC restriction of T cells is not clearly defined.^{42,43,45,46} Our study of the transcription of the TRG γ and TCR β genes indicates that these two groups of genes are expressed at all stages of thymic differentiation but that expression of full-length TCR β and TCR α mRNA tends to be high only in the more mature cases (CD3+). As opposed to Furley et al,³⁸ we did observe cases that, although rearranged, did not express detectable TCR β transcripts. Case 17 seems exceptional because the CD3 molecule was detected at the cell surface whereas only TCR β mRNA of 1.0 kb was found. Indeed, it has been shown that in T cells committed to express the CD3-Ti complex the CD3 molecule cannot be expressed at the cell surface without coexpression of the dimer Tia β .⁴⁷ A particular population of CD3+ T cells has recently been identified in the lymphocytes of immunodeficient patients²⁰ and in human thymocytes.²³ These Ti-negative cells coexpress the CD3 molecule with two other polypeptide chains whose molecular weights have been estimated at 55 and 40 kD in one of the reports²⁰ and at 62 and 44 kD in the other.²³ In one of these studies,²⁰ the use of antiserum directed against synthetic peptides V γ and C γ would suggest that one of these two chains is the product of TRG γ . The TRG γ -CD3 complex could represent a second antigen receptor on T cells. As in our case no. 17, one of the groups did find strong expression of TRG γ mRNA, a CD3+/CD4-/CD8- phenotype, and the exclusive presence of nonfunctional 1.0-kb TCR β mRNA in a clone of thymocytes. This case of T-ALL, as in the PEER cell line,⁴⁸ could thus possibly be a malignancy developing from such cells. The relation between these cells and the CD3+/CD4-/CD8- subset of peripheral T cells⁴⁹ and some subset of NK cells⁵⁰ remains to be established.

NOTE ADDED IN PROOF

In addition to the J γ P, J γ 1, and J γ 2 joining regions, two other J γ segments were recently described (Quertermous et al: *J Immunol* 138:2687, 1987). In case no. 5, we have found that the rearrangement involves the J segment located between C γ 1 and J γ 2.

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Thromboxane A₂ Causes Feedback Amplification Involving Extensive Thromboxane A₂ Formation on Close Contact of Human Platelets in Media With a Low Concentration of Ionized Calcium

By Marian A. Packham, Raelene L. Kinlough-Rathbone, and J. Fraser Mustard

Close platelet-to-platelet contact induced by weak agonists in a medium with a low concentration of Ca²⁺ leads to thromboxane A₂ (TXA₂) formation, release of granule contents, and secondary aggregation. These responses do not occur in a medium containing Ca²⁺ in the physiological range (1 to 2 mmol/L). Experiments were done to determine whether feedback amplification is required to generate amounts of TXA₂ that are sufficient to cause secondary aggregation and the reactions associated with it, or whether close platelet-to-platelet contact alone is sufficient to generate enough TXA₂ to produce these responses. Platelets were washed and resuspended in a modified Tyrode solution to which no calcium salt was added that contained 0.35% albumin and apyrase. This medium contains 20 μmol/L Ca²⁺ and 1 mmol/L Mg²⁺. Platelets were aggregated with adenosine diphosphate (ADP) in the presence of fibrinogen, agglutinated with polylysine, or after pretreatment with chymotrypsin, aggregated with fibrinogen. In the low-Ca²⁺ medium, all these agonists caused platelets to adhere to each other, followed by secondary aggregation with TXA₂ formation

and release of granule contents. When Ca²⁺ (1 to 2 mmol/L), aspirin, or the thromboxane receptor blocker BM 13.177 was present, the secondary responses did not occur; dazoxiben decreased thromboxane formation, but did not prevent secondary aggregation or release. Aspirin-treated platelets were less responsive to ADP, U46619, or TXA₂ in the low-Ca²⁺ medium, which indicated that the secondary responses of untreated platelets were not caused by a generalized increase in sensitivity. The reactions that result from close platelet-to-platelet contact in a low-Ca²⁺ medium can be caused by a wide variety of weak agonists; the secondary aggregation response and release of granule contents are dependent on TXA₂ formation and on feedback amplification by TXA₂ or the prostaglandin endoperoxides. The secondary responses caused by weak agonists in citrated platelet-rich plasma (which has a concentration of Ca²⁺ similar to the low-Ca²⁺ medium used in the present studies) do not occur at the concentration of Ca²⁺ in circulating blood and thus may have little biologic relevance.

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DURING ADENOSINE DIPHOSPHATE (ADP)-induced aggregation of human platelets, granule contents are released if the platelets are in a medium with a low concentration of ionized calcium (Ca²⁺) such as citrated platelet-rich plasma or if they are suspended in a medium such as a modified Tyrode solution to which no Ca²⁺ has been added.¹⁻⁶ This action of ADP requires the platelets to come into close contact with each other⁷⁻⁹ and involves activation of the reactions that lead to the formation of thromboxane A₂ (TXA₂).^{9,10} Platelets can be brought together by other agents such as the agglutinating agent polylysine^{3,11} or by fibrinogen when the surface of platelets has been modified by pretreatment with chymotrypsin¹²; when this is done in a medium with a low concentration of Ca²⁺, the contents of platelet granules are released.^{3,11}

Since close platelet-to-platelet contact in a medium with a low concentration of Ca²⁺ appears to induce the release reaction through activation of the arachidonate pathway, which leads to TXA₂ formation, we have carried out experiments to determine whether feedback amplification is required to generate amounts of TXA₂ that are sufficient to cause secondary aggregation and the reactions associated with it or whether close platelet-to-platelet contact alone is sufficient to generate enough TXA₂ to produce these responses. We studied aggregating and agglutinating agents that do not activate the arachidonate pathway or cause the release reaction when the platelets are suspended in media containing a concentration of Ca²⁺ in the physiological range (1 to 2 mmol/L) and the effects of a cyclooxygenase inhibitor (aspirin), a thromboxane synthetase inhibitor (dazoxiben), and a thromboxane receptor blocker (BM 13.177).

MATERIALS AND METHODS

Materials were obtained from the following suppliers: ADP, aspirin, chymotrypsin (lot no. 109C-8015), human thrombin, hepa-

rin (grade 1), poly-DL-lysine hydrobromide (approximate molecular weight of 35,000), arachidonic acid, and imipramine were from Sigma Chemical Co, St Louis. Albumin (Pentex fraction V) was from Miles Laboratories, Elkhart, IN. U46619, a prostaglandin endoperoxide analogue, was from the Upjohn Co, Kalamazoo, MI. BM 13.177, a TXA₂ receptor blocker, was a generous gift from Dr K. Stegmeier, Boehringer Mannheim, FRG. Dazoxiben (UK-37,248-01) (a thromboxane synthetase inhibitor) was from Pfizer, Sandwich, Kent, UK. Human fibrinogen (grade L, AB Kabi, Stockholm) was treated with diisopropylfluorophosphate (Sigma) before use to inhibit serine proteases¹ and was partially purified by the method of Lawrie et al.¹³ Apyrase, prepared from potatoes by the method of Molnar and Lorand,¹⁴ was dissolved in 0.15 mol/L NaCl and stored at -20°C. ¹⁴C-serotonin (as 5-hydroxytryptamine-3'-¹⁴C-creatinine sulphate, 50 mCi/mmol) and ¹⁴C-arachidonic acid (>50 mCi/mmol) were from Amersham Corp, Oakville, Ontario, Canada, and a radioimmunoassay kit for thromboxane B₂ (TXB₂) was from NEN-Lachine, Quebec.

Washed human platelets were prepared as previously

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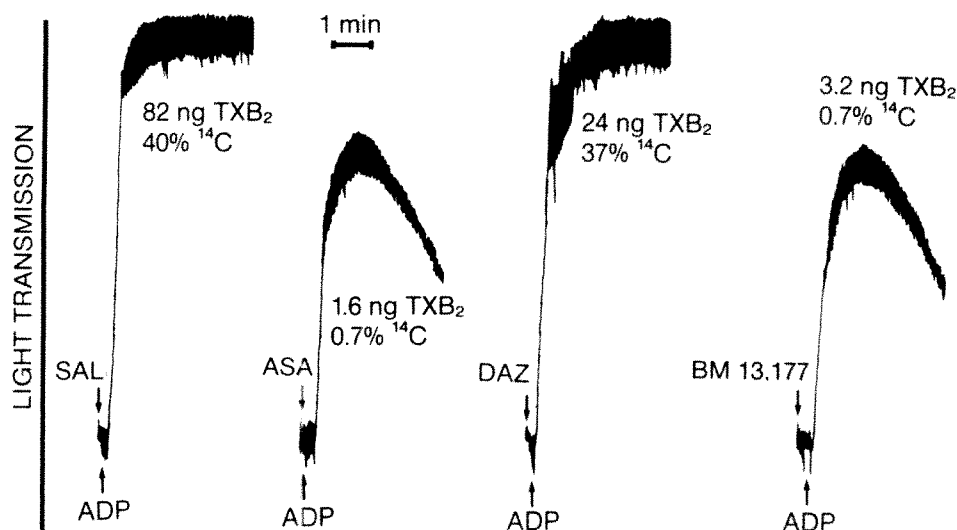


Fig 1. Effect of aspirin (ASA, 100 μ mol/L), dazoxiben (DAZ, 100 μ mol/L), or BM 13.177 (100 μ mol/L) on ADP-induced aggregation, formation of TXB₂ (ng/10⁹ platelets), and release of ¹⁴C-serotonin (percentage of ¹⁴C) from washed human platelets suspended in a modified Tyrode-albumin solution with no added Ca²⁺. Fibrinogen (400 μ g/mL) was added before ADP (10 μ mol/L); a 0.85% NaCl solution (SAL) was added in the experiment without an inhibitor to maintain a constant volume. Results are typical of four experiments.

described.^{3,15} The final suspending medium was a modified Tyrode solution from which calcium was omitted; it contained 1 mmol/L Mg²⁺, apyrase at a concentration that converted 0.25 μ mol/L ATP to adenosine monophosphate in 120 seconds at 37°C, and albumin (0.35%). The concentration of Ca²⁺ in the medium that contained the platelets was approximately 20 μ mol/L as measured by atomic absorption spectrophotometry. This medium is termed the low-Ca²⁺ medium.

The platelet count was adjusted to 500,000/ μ L. Platelet aggregation was studied at 37°C in an aggregation module (Payton Associates, Scarborough, Ontario). Platelets were labeled in the first washing solution with ¹⁴C-serotonin (2 μ Ci/10 mL washing fluid). Release of serotonin was measured as described previously.¹⁶ Imipramine (5 μ mol/L) was used to inhibit the reuptake of released serotonin.

TXB₂ was measured in the supernatant samples prepared by rapid centrifugation of platelet suspensions for one minute at 12,000 g in an Eppendorf centrifuge (Brinkmann, Rexdale, Ontario).

In some experiments platelets were treated in the second washing solution with chymotrypsin (10 U/mL).¹² For studies with ¹⁴C-arachidonic acid, platelets were labeled in the first washing fluid with 0.1 μ Ci/mL.

RESULTS

In the presence of fibrinogen, ADP-induced aggregation of human platelets suspended in a medium with a low

concentration of Ca²⁺ led to second-phase aggregation, the formation of TXB₂, and release of ¹⁴C-serotonin, all of which could be inhibited by pretreating the platelets with aspirin (Figs 1 and 2). Release of ¹⁴C-serotonin and appreciable TXB₂ formation did not occur until the beginning of the second phase of aggregation. The addition of dazoxiben, an inhibitor of thromboxane formation, did not prevent the ADP-induced release of ¹⁴C-serotonin or the enhanced aggregation in this medium; it did decrease the amount of TXB₂ that was detected. In contrast, blocking the thromboxane receptor with BM 13.177 prevented the release of ¹⁴C-serotonin and inhibited TXB₂ formation caused by ADP. In these experiments, the concentrations of aspirin or the thromboxane receptor blocker that were used completely prevented aggregation and release induced by 10 to 100 μ mol/L arachidonic acid; aspirin also prevented TXB₂ formation from arachidonic acid, but BM 13.177 (100 μ mol/L) did not.

The addition of Ca²⁺ (1 or 2 mmol/L) to the platelet-suspending medium prevented ADP-induced secondary aggregation, TXB₂ formation, and release of ¹⁴C-serotonin (Fig 2). The extent of primary-phase aggregation was greater in the medium with 2 mmol/L Ca²⁺; primary aggregation was followed by deaggregation.

Fig 2. Effect of Ca²⁺ (1 or 2 mmol/L) on the response of platelets to ADP (10 μ mol/L) in the presence of fibrinogen (400 μ g/mL). Washed human platelets were suspended in a modified Tyrode-albumin solution with no added Ca²⁺. In the medium without Ca²⁺, a 0.85% NaCl solution (SAL) was added to maintain a constant volume. The amounts of TXB₂ formed (ng/10⁹ platelets) and ¹⁴C-serotonin released (percentage of ¹⁴C) are shown beside the aggregation curves. Similar results were obtained with 5 μ mol/L ADP. Results are typical of four experiments.

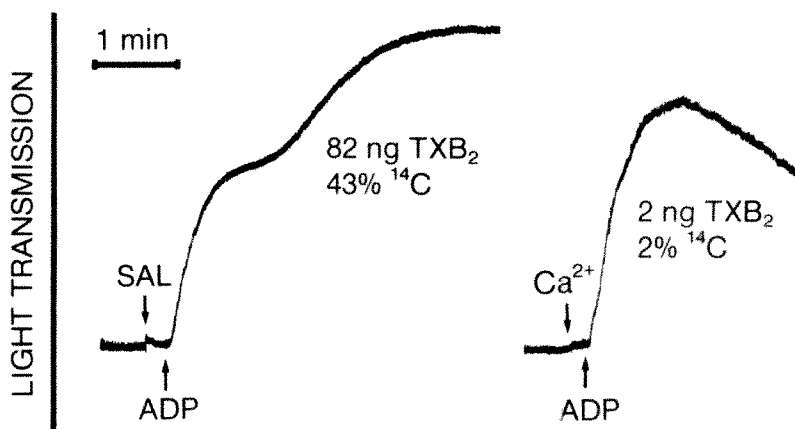
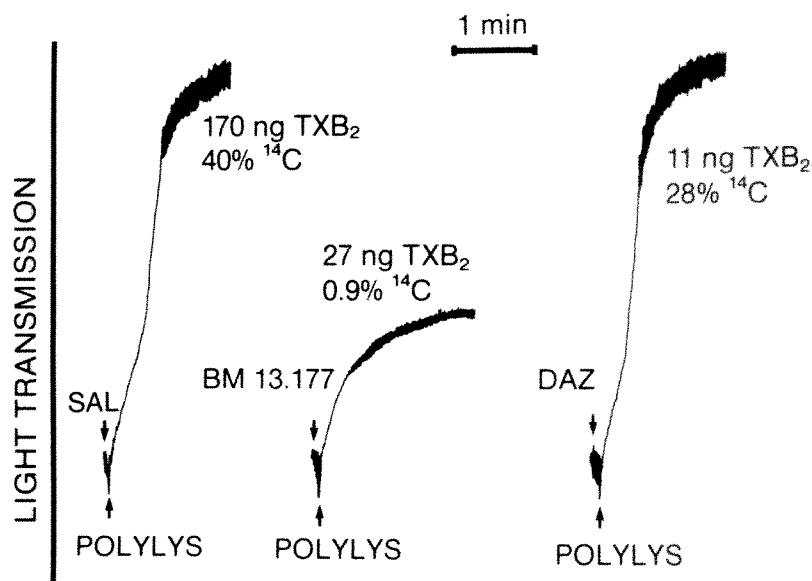


Fig 3. Effect of BM 13.177 (100 $\mu\text{mol/L}$) or dazoxiben (DAZ, 100 $\mu\text{mol/L}$) on the response of platelets to polylysine (10 $\mu\text{g/mL}$) in a modified Tyrode-albumin solution with no added Ca^{2+} . When no inhibitor was added, a 0.85% NaCl solution (SAL) was added to maintain a constant volume. The amounts of TXB_2 formed (ng/ 10^9 platelets) and ^{14}C -serotonin released (percentage of ^{14}C) are shown beside the agglutination/aggregation curves. Similar results were obtained with 20 and 40 $\mu\text{g/mL}$ polylysine. Results are typical of four experiments.



Polylysine-induced agglutination of platelets suspended in a low- Ca^{2+} medium was followed by extensive aggregation accompanied by the formation of TXB_2 and the release of ^{14}C -serotonin (Fig 3). These secondary effects were inhibited by the addition of the TXA_2 receptor blocker BM 13.177; dazoxiben inhibited TXB_2 formation, but did not prevent the release of ^{14}C -serotonin or secondary aggregation induced by polylysine (Fig 3). Pretreatment of the platelets with aspirin (100 $\mu\text{mol/L}$) or the addition of Ca^{2+} (2 mmol/L) inhibited TXB_2 formation and release of ^{14}C -serotonin (aspirin, 4.3 ng TXB_2 / 10^9 platelets, 2.6% release; Ca^{2+} , 3.3 ng TXB_2 / 10^9 platelets, 3.0% release); secondary aggregation was blocked under these conditions.

The addition of fibrinogen to chymotrypsin-treated human platelets suspended in a low- Ca^{2+} medium induced secondary aggregation, formation of TXB_2 , and the release of ^{14}C -serotonin (Fig 4). These secondary effects were blocked by pretreating the platelets with aspirin or by adding the thromboxane receptor blocker BM 13.177. In the presence of these inhibitors, fibrinogen induced only the primary phase of aggregation (Fig 4). The addition of Ca^{2+} (2 mmol/L) to the suspending medium inhibited secondary

aggregation, TXB_2 formation, and release of ^{14}C -serotonin (1.2 ng TXB_2 / 10^9 platelets, 2.4% release).

For the purpose of comparison, the amount of TXB_2 formed upon stimulation of platelets with thrombin, a strong agonist, was determined. With concentrations of thrombin ranging from 0.5 to 5 U/mL, the mean value for TXB_2 formation was $1,530 \pm 440$ ng/ 10^9 platelets (mean $\pm$ SD of 13 determinations with platelets from five different subjects).

To determine whether platelets are more sensitive to TXA_2 in a medium without added Ca^{2+} , we compared the effect of the endoperoxide mimetic U46619 (0.5 and 1 $\mu\text{mol/L}$) on platelets pretreated with aspirin and suspended in media with or without added Ca^{2+} . Although there was little or no difference in the rate or extent of aggregation (except when release was less than 5%), the amount of ^{14}C -serotonin released from prelabeled platelets was much less from the platelets suspended in a medium without added Ca^{2+} than from platelets suspended in a medium containing 2 mmol/L Ca^{2+} (Table 1). There was practically no difference between results with 1 mmol/L Ca^{2+} and those with 2 mmol/L Ca^{2+} (not shown).

Fig 4. Effect of aspirin (ASA, 100 $\mu\text{mol/L}$) or BM 13.177 (100 $\mu\text{mol/L}$) on the response of chymotrypsin-pretreated platelets to fibrinogen (Fbg, 150 $\mu\text{g/mL}$) in modified Tyrode-albumin solution with no added Ca^{2+} . When no inhibitor was added, a 0.85% NaCl solution (SAL) was added to maintain a constant volume. The amounts of TXB_2 formed (ng/ 10^9 platelets) and ^{14}C -serotonin released (percentage of ^{14}C) are shown beside the aggregation curves. Similar results were obtained with 230 $\mu\text{g/mL}$ fibrinogen. Results are typical of three experiments.

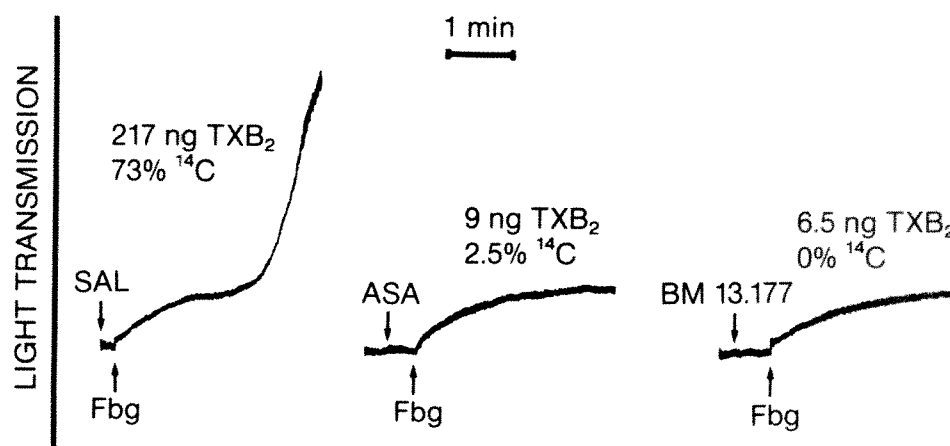


Table 1. Effect of the Concentration of Ca^{2+} in the Suspending Medium on the Release of ^{14}C -Serotonin Induced by U46619 From Human Platelets Pretreated With Aspirin

Experiment	U46619 ($\mu\text{mol/L}$)	Percent Release of ^{14}C -Serotonin	
		With Ca^{2+} (2 mmol/L)	Without Added Ca^{2+}
1	1.0	52	43
	0.5	46	32
2	1.0	32	24
	1.0	22	13
3	0.5	7	3
	1.0	11	0
4	1.0	20	14
	0.5	11	8
5		8	3

Platelets in the first washing fluid were incubated with 500 $\mu\text{mol/L}$ aspirin. For paired difference analysis of samples with and without added Ca^{2+} , $P < .001$.

In a second approach to investigating the possibility that platelets might be more sensitive to TXA_2 in a medium with a low concentration of Ca^{2+} , aspirin-treated, ^{14}C -serotonin-labeled platelets were mixed with untreated, unlabeled platelets, and the mixture was stimulated with arachidonic acid (200 $\mu\text{mol/L}$). In the low- Ca^{2+} medium, less ^{14}C -serotonin was released (13%) than in the medium with 2 mmol/L Ca^{2+} (17%) (mean of two experiments).

The following experiment was done to investigate the possibility that unstimulated platelets in a medium without added Ca^{2+} mobilized more arachidonic acid than platelets in a medium with a more physiological concentration of Ca^{2+} . Washed human platelets were prelabeled with ^{14}C -arachidonic acid and incubated in a medium with either 2 mmol/L Ca^{2+} or without added Ca^{2+} . Over a four-hour period at 37°C there was no difference in the rate or extent of accumulation of radioactivity in the suspending fluid.

DISCUSSION

These experiments were done with agonists that do not activate the arachidonate pathway or cause release of granule contents in the presence of concentrations of Ca^{2+} in the physiological range. The results demonstrate that close contact between platelets suspended in a medium without added Ca^{2+} (approximately 20 $\mu\text{mol/L}$ Ca^{2+}) at 37°C activates the arachidonate pathway and leads to TXA_2 formation, the release of granule contents, and secondary aggregation. The primary wave of aggregation or agglutination does not involve the release of granule contents or thromboxane formation, but the close contact during primary aggregation in such a medium induces a second wave of aggregation that is attributable to the formation of TXA_2 and the release of granule contents, particularly ADP.^{3,9} This activation in the low- Ca^{2+} medium is not agonist specific since, as shown in the present experiments and in earlier studies,^{1,3,11,15} it can be brought about by ADP in the presence of fibrinogen, by polylysine, or by the addition of fibrinogen to platelets that have been pretreated with chymotrypsin.¹² We¹⁷ and others¹⁸ have shown similar effects when close platelet contact in a low- Ca^{2+} medium has been caused by ADP in the presence of

von Willebrand factor, asialo-von Willebrand factor,¹⁹ epinephrine in the presence of fibrinogen,²⁰ or centrifugation.^{7,8} None of these agonists or treatments causes TXA_2 formation, release of granule contents, or secondary aggregation in a medium containing 1 to 2 mmol/L Ca^{2+} . Close platelet-to-platelet contact is essential for activation by these agents in the low- Ca^{2+} medium, and they can therefore be classified as weak agonists according to the definition of Detwiler and Huang.⁹ They differ from strong agonists such as thrombin or the divalent cation ionophore A23,187 in that strong agonists can cause TXA_2 formation and the release of granule contents without aggregation, and strong agonists cause TXA_2 formation and release in media containing high concentrations of Ca^{2+} (1 to 2 mmol/L); the amounts of TXA_2 formed by platelets in response to high concentrations of thrombin (0.5 to 5 U/mL) are approximately ten times greater than the amounts formed upon close platelet-to-platelet contact induced by a weak agonist in the low- Ca^{2+} medium. Aggregation and release induced by strong agonists is not blocked by inhibitors of cyclooxygenase such as aspirin or indomethacin.¹⁵ In addition, thromboxane receptor blockers such as BM 13,177 prevent second-phase aggregation induced by weak agonists when they induce close platelet contact in a low- Ca^{2+} medium, whereas thromboxane receptor blockers do not inhibit aggregation induced by strong agonists.²¹

It is apparent that the reactions that result from close platelet-to-platelet contact in the low- Ca^{2+} medium are dependent upon the formation of TXA_2 or the prostaglandin endoperoxides since in all cases they can be blocked by aspirin. Strong inhibition of secondary aggregation and ^{14}C -serotonin release by the thromboxane receptor blocker BM 13,177 indicates that feedback amplification by TXA_2 or by the prostaglandin endoperoxides is required for the formation of large amounts of TXA_2 , release of granule contents, and secondary aggregation. It is evident that the prostaglandin endoperoxides can substitute for TXA_2 to a large extent since the thromboxane synthetase inhibitor dazoxiben partially inhibited but did not prevent the release of granule contents and did not prevent secondary aggregation.

Activation of the reactions leading to thromboxane formation upon close platelet-to-platelet contact in the low- Ca^{2+} medium is not attributable to the freeing of a larger amount of arachidonate than in the high- Ca^{2+} medium, as shown by experiments with unstimulated platelets prelabeled with ^{14}C -arachidonic acid. Thus it is not the low- Ca^{2+} concentration per se, but close platelet-to-platelet contact in the low- Ca^{2+} medium that is responsible for activation of the pathway leading to TXA_2 formation. In addition, it is evident from the experiments with BM 13,177 that in the low- Ca^{2+} medium, more arachidonate is not mobilized when feedback amplification by TXA_2 is blocked, because the amount of TXB_2 detected after stimulation with ADP in the presence of BM 13,177 was minimal and was not appreciably greater than the amount detected in the medium with 2 mmol/L Ca^{2+} .

Results from three types of experiments indicate that platelets are not intrinsically more sensitive to stimulation in

a low- Ca^{2+} medium, although the formation of TXA_2 upon close contact makes them appear to be more responsive: (a) in the low- Ca^{2+} medium, release of ^{14}C -serotonin from aspirin-treated platelets in response to U46619 was significantly less than in the media with 1 or 2 mmol/L Ca^{2+} ; (b) when aspirin-treated, ^{14}C -serotonin-labeled platelets were mixed with unlabeled platelets and the mixture was stimulated with arachidonic acid, less ^{14}C -serotonin was released than in the medium with 2 mmol/L Ca^{2+} , thereby indicating that the platelets are less sensitive to TXA_2 in the low- Ca^{2+} medium; and (c) primary aggregation in response to ADP in the presence of fibrinogen was less extensive in the low- Ca^{2+} medium.

These reactions upon close platelet-to-platelet contact in a low- Ca^{2+} medium are not seen with platelets from all species. They have been observed with platelets from most other primates, cats, piebald and rosette guinea pigs, and some dogs but not with platelets from rabbits, rats, mice, pigs, mink, cows, goats, sheep, or most dogs and horses.²² In these latter species, ADP does not cause secondary aggregation in citrated platelet-rich plasma even if high concentrations of ADP are used. The reason for this species difference is not apparent.

It should be emphasized that the platelet-suspending medium used in the present experiments contained 1 mmol/L Mg^{2+} and approximately 20 $\mu\text{mol/L}$ Ca^{2+} . The reactions do not occur if all the Ca^{2+} is chelated with ethylene glycol bis-(β -aminoethyl ether) N,N,N',N'-tetraacetic acid or all the divalent cations are chelated with EDTA.³ The conditions leading to secondary aggregation in the low- Ca^{2+} medium are similar to those in citrated platelet-rich plasma, which contains approximately 40 $\mu\text{mol/L}$ Ca^{2+} .⁶ When blood is taken into hirudin as the anticoagulant, the concentration of Ca^{2+} is about 1.1 mmol/L, and ADP either does not induce the release of granule contents^{1,6} or induces much less release than in citrated platelet-rich plasma.⁶ Since the concentration of Ca^{2+} in circulating blood is about 1 mmol/L,⁴ it is unlikely that the close platelet contact phenomenon leading to thromboxane formation and the release of granule contents that has been so extensively studied in citrated platelet-rich plasma has any biologic relevance.

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¹¹¹In-Oxine Platelet Survivals in Thrombocytopenic Infants

By Valerie Castle, Geoffrey Coates, John G. Kelton, and Maureen Andrew

Thrombocytopenia is a common occurrence (20%) in sick neonates, but the causes have not been well studied. In this report we demonstrate that thrombocytopenia in the neonate is characterized by increased platelet destruction as shown by shortened homologous ¹¹¹In-oxine-labeled platelet life spans. Thirty-one prospectively studied thrombocytopenic neonates were investigated by measuring the ¹¹¹In-labeled platelet life span, platelet-associated IgG (PAIgG), and coagulation screening tests. In every infant, the thrombocytopenia was shown to have a destructive component since the mean platelet life span was significantly shortened to 65 ± 6 (mean $\pm$ SEM) hours with a range of one to 128 hours compared with adult values (212 ± 8 ; range, 140 to 260; gamma function analysis). The platelet survival was directly related to the lowest platelet count and inversely related to both the highest mean platelet volume and duration of the thrombocytopenia. In

22 infants the percent recovery of the radiolabeled platelets was less than 50%, which suggested that increased sequestration also contributed to the thrombocytopenia. Infants with laboratory evidence of disseminated intravascular coagulation ($n = 8$) or immune platelet destruction evidenced by elevated levels of PAIgG ($n = 13$) had even shorter platelet survivals and a more severe thrombocytopenia compared with the ten infants in whom an underlying cause for the thrombocytopenia was not apparent. Full-body scintigraphic images obtained in 11 infants showed an increased uptake in the spleen and liver, with a spleen-to-liver ratio of 3:1. This study indicates that thrombocytopenia in sick neonates is primarily destructive, with a subgroup having evidence of increased platelet sequestration.

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THROMBOCYTOPENIA occurs frequently (20%) in sick neonates admitted to a neonatal intensive care unit.¹ The mechanism responsible for the thrombocytopenia has been the subject of controversy, with some investigators suggesting that decreased platelet production is the cause of the thrombocytopenia, whereas others have reported that increased platelet destruction is the dominant mechanism.^{2,3} In a previous study, we postulated that the thrombocytopenia in neonates was caused by an increased rate of platelet destruction.¹ However, these conclusions were primarily based upon measurements of platelet volume and assessments of bone marrow megakaryocytes mass and response to platelet transfusions. Although all are consistent and compelling, they remain indirect measures. The benchmark for classifying the mechanism of thrombocytopenia into underproduction, sequestration, and increased destruction is the performance of radiolabeled platelet survival studies. Until recently, such studies could not be performed in infants primarily because of the low specific activity of the traditional platelet label, Chromium 51. The recent application of ¹¹¹In-oxine, which has a high labeling efficiency for platelets, now allows the performance of platelet survival studies in infants when using very small volumes of sample (<1 mL). In addition, this isotope emits medium-energy γ -rays with high abundance, which makes organ imaging possible. In

this report we describe the application of homologous ¹¹¹In-labeled platelet survival studies in the investigation of thrombocytopenic infants. We found that all thrombocytopenic infants studied had shortened platelet life spans when compared with adults and that in some patients platelet sequestration occurred.

MATERIALS AND METHODS

Patient Population

Thrombocytopenic infants less than 2 months of age with a platelet count less than $100 \times 10^9/L$ admitted to the McMaster Regional Neonatal Intensive Care Unit were eligible for this study. Patients were entered into the study if informed consent was obtained and the infant was in stable condition. The study was approved by the university-approved Ethics Review Committee and Nuclear Physics Review Committee.

A data base was collected for each infant, and the information included Apgar scores, the presence of respiratory distress syndrome (RDS), evidence of infection, umbilical lines, exchange transfusions, antibiotics, and maternal drug use. The gestational age was determined by a combination of maternal dates and Dubowitz scores.⁴

Investigations

Platelet life span studies. Random donor platelets obtained from the Canadian Red Cross were used for the performance of the platelet survival studies. Platelets were obtained from an ABO-compatible, hepatitis B surface antigen-negative, unrelated donor. After centrifugation and washing with acid-citrate-dextrose (ACD), the platelets were resuspended with 30 to 50 μ Ci of ¹¹¹In-oxine and incubated at room temperature for 30 minutes. The labeled platelets were then recentrifuged with ACD and subsequently resuspended in platelet-poor plasma. Each infant received 8 to 15 μ Ci/kg of ¹¹¹In-labeled platelets (2 to 4 mL), the higher dose used for organ imaging. The labeled platelets were injected into a peripheral intravenous line and blood sampling performed at 1, 6, 8, 12, 24, 48, 72, and 96 hours after injection. The blood samples were obtained by microtechniques and consisted of 0.5 mL of whole blood collected into EDTA. The ¹¹¹In radioactivity in the samples was measured in a gamma scintillation well counter (1085 Nuclear Chicago Gamma Counter, Chicago), and platelet survival curves were constructed. The survival curves were fitted to linear, exponential, and gamma functions by using the least-squares method and a digital computer.

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The gamma function was constructed from the multiple-hit program developed by Murphy and Francis, and this was used for all calculations of platelet survival.⁵ Radioactivity in the liver and spleen was expressed as a function of the radioactivity in the whole body by using appropriate computer-generated regions of interest. We calculated the percent recovery of injected platelets in the one-hour sample as follows (assuming a blood volume of 80 mL/kg): percent recovery = (cpm/mL) $\times$ total blood volume/injected dose. Imaging was performed by using a gamma scintillation camera with a large field of view.

Laboratory investigations. The laboratory investigations performed in all infants included platelet counts, measurements of the mean platelet volume (MPV) and platelet-associated IgG (PAIgG), and coagulation assays. All blood samples were obtained from nonheparinized umbilical catheters or antecubital veins. The platelet counts and MPV were measured on EDTA-anticoagulated specimens by using a Coulter-S+ Counter (Coulter Electronics, Hialeah, FL), with confirmation by examination of a stained blood film. PAIgG was measured on 2 mL of whole blood collected into ACD. The platelets were isolated and washed and the total PAIgG (lysed platelets) measured by using an immunoradiometric assay.⁶ The coagulation assays were performed by microtechniques⁷ on blood collected into polypropylene tubes containing 3.8% sodium citrate and 0.1 mol/L ϵ -aminocaproic acid. The prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin clotting time (TCT), and fibrinogen level were measured on all samples. Abnormal results, (greater or lower than 2 SD of the age-matched normal range) led to further investigations including measurement of factors V and VIII procoagulant (VIII:C), and fibrinogen-fibrin degradation products (FDP, Thrombo-Welco Test; General Diagnostics, Morris Plains, NJ).⁸ The diagnosis of disseminated intravascular coagulation (DIC) required prolongation of the screening tests plus the presence of elevated levels of FDP or low levels of factors V and VIII:C and fibrinogen.

Differences between groups were analyzed by Student's unpaired *t* test. For multiple comparisons the Bonferroni correction was used. The relationship between two variables was analyzed by using linear regression. Probability values $<.05$ or $<.01$ (as indicated) were considered significant.

RESULTS

Patient Population

The clinical characteristics of the 31 infants studied are shown in Table 1. The infants are grouped as either premature or full term because of the differing spectrum of disorders present in these patients. A five-minute Apgar score <7 was present in 12 infants. Twenty-five infants

Table 1. Clinical Characteristics of the Thrombocytopenic Infants

Characteristic	Premature	Full-Term
Number	21	10
Gestational age (wk)*	30 $\pm$ 3.8	39.5 $\pm$ 1.5
Birth weight (kg)*	1.460 $\pm$ 0.738	3.143 $\pm$ 0.765
5-min Apgar score (<7)†	9	3
IPPV†	20	5
RDS†	20	1
Positive blood culture†	3	0
Positive viral culture†	2	2

Abbreviation: IPPV, intermittent positive pressure ventilation.

*Mean $\pm$ 1 SD.

†Reported as the number of infants affected.

required assisted ventilation. Three infants had positive blood cultures, all with *Propionobacterium*, and four infants had viral infections documented by culture (three with cytomegalovirus and one with an enterovirus [Echo 11]).

Platelet Survival and Imaging

All 31 infants studied had evidence of a destructive thrombocytopenia as demonstrated by a shortened platelet life span compared with adults. The mean gamma function life span in the infants was 65 $\pm$ 6 hours (mean $\pm$ SEM) with a range of 1 to 128 hours compared with 212 $\pm$ 8 hours in the adult. There was a correlation between the lowest platelet count for each infant and platelet survival ($r = .40$; $P = .02$; platelet count = 16.704 + 0.338 $\cdot$ platelet survival; Fig 1). There was an inverse relationship as well between both the duration of the thrombocytopenia and platelet survival ($r = -.63$; $P < .01$; duration = 124.825 - 55.8600 $\cdot$ log platelet survival) and between the highest MPV and platelet survival ($r = -.42$; $P < .05$; MPV = 15.4976 - 1.9696 $\cdot$ log platelet survival).

The immediate percent recovery of the radiolabeled platelets was $>50\%$ (the lower limit for the normal adult) in nine infants; however, in 22 infants there was a percent recovery $<50\%$ (24% $\pm$ 3.4, mean $\pm$ SEM) that suggested increased platelet sequestration. Platelet turnover was calculated for each infant by using a standard approach,⁹ and only three infants had definite evidence of decreased production contributing to their thrombocytopenia. Organ imaging was performed in 11 infants who were sufficiently stable to be transported to the nuclear medicine department. All 11 had a low percent recovery, with 13% $\pm$ 1.7% (range, 5% to 18%) uptake in the liver and 40% $\pm$ 4.9% (28% to 54%) uptake in the spleen, for a spleen-to-liver ratio of approximately 3:1. Two of the 11 infants had abnormal sites of accumulation: one infant had increased uptake in the lung and scalp; the other infant had increased uptake in the posterior fossa. The

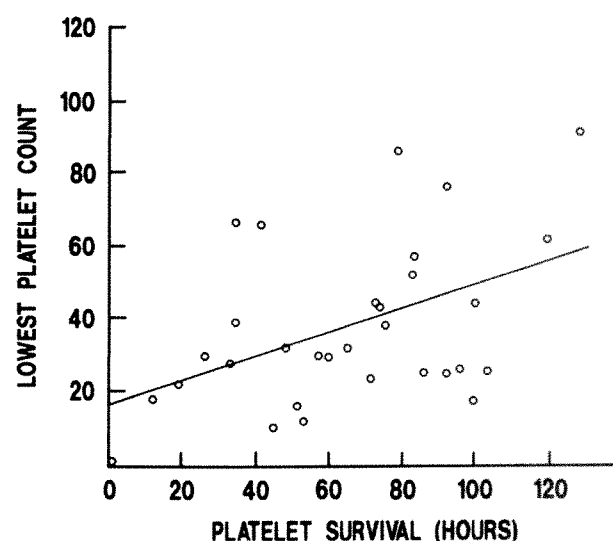


Fig 1. The relationship between the lowest platelet count and platelet survival in 31 thrombocytopenic infants ($r = .40$; $P = .02$; platelet count = 16.704 + 0.338 $\cdot$ platelet survival).

Table 2. Laboratory Characteristics of the Thrombocytopenic Infants

Group	n	PT (s)	APTT (s)	TCT* (s)	Fibrinogen (g/L)	PAIgG (fg/Platelet)
PAIgG	13	14 ± 0.4†	46 ± 2.7	24 ± 0.8	2.2 ± 0.19	18.0 ± 2.80
DIC	8	23 ± 2.8	95 ± 11.4	36 ± 2.8	1.2 ± 0.16	3.7 ± 0.47
Neither	10	14 ± 0.6	53 ± 2.5	24 ± 1.0	3.9 ± 0.45	3.4 ± 0.53

The three groups were defined on the basis of elevated PAIgG levels (normal is less than 5.5 fg/platelet), the presence of DIC, or neither.

*Two-unit TCT.

†Mean ± 1 SEM.

cause for the abnormal sites of accumulation was uncertain but may have been due to active bleeding in these areas.

Laboratory Investigations

Further evidence that the thrombocytopenia was due to increased platelet destruction was provided by the demonstration of an increased MPV in all infants (12.3 ± 0.4 fL, normal <10.0 fL) plus a poor ($<30 \times 10^9/L$) incremental response to random donor platelets in 13 of 24 infants. The cause for the increased platelet destruction was classified as immune (based upon the presence of PAIgG), DIC, or neither. Eight infants had evidence of DIC, 13 had increased PAIgG levels, and ten had neither DIC nor increased PAIgG levels (Table 2). Infants for whom either DIC or increased PAIgG levels were present had a higher MPV, a lower

platelet nadir, and a longer duration of thrombocytopenia (Fig 2). In addition, on subanalysis the infants with either DIC or increased PAIgG levels had a significantly shorter platelet life span compared with infants in whom neither DIC nor increased PAIgG levels were present (Fig 3).

DISCUSSION

The systematic application of platelet survival studies to thrombocytopenic adults has increased our understanding of the pathophysiology of a variety of thrombocytopenic disorders. The classification of neonatal thrombocytopenias also would benefit by the performance of such studies. In this report we describe the use of ^{111}In -oxine for the performance of platelet survival studies plus organ imaging in 31 neonates with thrombocytopenia. The shortened platelet survivals observed correlated with the basic mechanisms responsible for the thrombocytopenia. Our results are compatible with a previous report of shortened platelet survival studies using the traditional label ^{51}Cr in 15 ill newborn infants, not all of whom were thrombocytopenic.³ The ^{51}Cr label has a number of limitations including a low labeling efficiency and the inability to quantitate platelet localization in various organs.

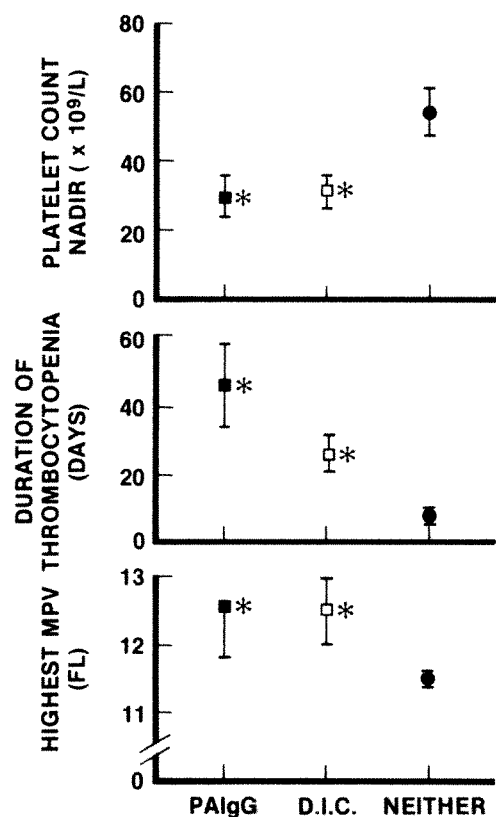


Fig 2. The lowest platelet count, duration of thrombocytopenia, and MPV in 31 thrombocytopenic infants (mean $\pm$ 2 SEM). Infants with either increased levels of PAIgG ($n = 13$) or DIC ($n = 8$) had a more severe thrombocytopenia with a longer duration and higher MPV compared with the infants ($n = 10$) in whom neither DIC nor increased PAIgG levels were present (* $P < .01$).

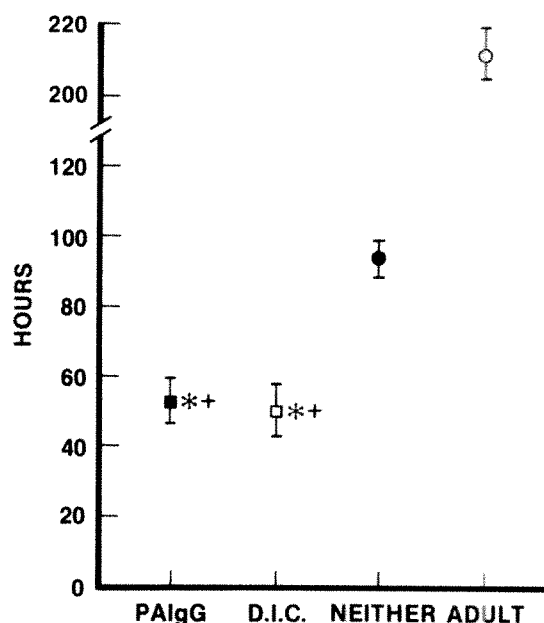


Fig 3. The results of ^{111}In -labeled platelet life span studies (mean $\pm$ 2 SEM) in 31 infants. Infants with either increased levels of PAIgG ($n = 13$) or DIC ($n = 8$) had shorter platelet survivals (* $P < .01$) compared with the thrombocytopenic infants ($n = 10$) without DIC or increased PAIgG levels and the adult (* $P < .01$).

The recent availability of ^{111}In -oxine as a platelet label overcomes many of these problems. ^{111}In has a high labeling efficiency (90%) that permits the radiolabeling of small volumes of platelets¹⁰⁻¹³; however, the volumes required were still too large to permit autologous platelet survival studies in the infant. The γ emission characteristics of ^{111}In -oxine allow this radiolabel to be used for organ imaging, which was performed for some of the infants in this report.

In a previous study, we had postulated that the mechanism of thrombocytopenia, which is seen in approximately 20% of ill neonates, was due to an increased rate of platelet destruction. This conclusion was based largely on the measurement of platelet volume, assessment of megakaryocyte mass, and response to platelet transfusions, which are all indirect measures of platelet life span. In the current study using ^{111}In -labeled platelet survival measurements, we confirmed our previous impressions and showed that thrombocytopenia in ill neonates was primarily due to an increased rate of platelet destruction. In three infants, underproduction based on the calculated platelet turnover also contributed to the thrombocytopenia.⁹ A fixed daily turnover alone could not account for the short ^{111}In -labeled platelet survivals; however, it may have contributed in part to the shortened survivals.¹⁴ The shortest platelet life spans were observed in infants with the lowest platelet count, highest MPV, and the longest duration of thrombocytopenia. In addition, a low percent recovery was observed for about two thirds of the infants, thereby suggesting that increased splenic sequestration could have contributed to the thrombocytopenia. The platelet life spans and percent recoveries in the thrombocytopenic neonates were compared with the same measurements in healthy adults. Ideally, the estimates of the platelet life span and platelet recoveries should have been compared with ^{111}In platelet studies performed in healthy, nonthrombocytopenic neonates. However, we did not perform such studies. Nonetheless, there is no reason to believe that the platelet life span in healthy neonates is different from that in adults with the identical normal range for the platelet count and the same platelet volume. In addition, we have recently shown that platelet life spans and percent recoveries using ^{111}In -labeled platelets are identical for neonatal rabbits compared with adult rabbits.¹⁵

In an attempt to correlate the platelet life span with the underlying cause of the thrombocytopenia, we grouped our patients into three groups: immune platelet destruction (evidenced by elevated levels of PAIgG), DIC (evidenced by abnormal coagulation test results consistent with increased

thrombin activity), and thrombocytopenic disorders not due to detectable immune mechanisms or DIC. As we found in our previous study, there was little overlap among these three groups, and the proportion of infants in each group was similar to what was found previously.¹ The infants with the thrombocytopenia in whom a cause for the platelet destruction could be found (DIC or elevated PAIgG levels) had significantly more severe thrombocytopenia compared with the infant in whom no cause could be found (Fig 2). Consistent with the severity of their thrombocytopenia was the demonstration of significantly shorter platelet life spans in these two groups compared with the thrombocytopenic infants with neither elevated PAIgG levels nor DIC (Fig 3).

One of the advantages of ^{111}In is the ability to perform quantitative in vivo imaging. In vivo distribution studies of ^{111}In -labeled platelets in the human healthy adult¹⁶⁻¹⁹ and healthy rabbit²⁰ have shown that the liver and spleen are the major sites of platelet deposition. Our study shows the same to be true in the infant with a predominant uptake in the spleen and liver. ^{111}In -labeled platelets have also been used in certain pathological circumstances to image abnormal accumulation of platelets in the human adult and in animals.²¹⁻²⁴ There has been only one report of the use of ^{111}In -labeled platelets in an infant, and this was to image a hemangioma in an infant with Kasabach-Merritt syndrome.²⁵ In our study, of the 11 infants who were imaged, two showed evidence of abnormal platelet accumulation. One very premature infant had an abnormal accumulation in the posterior occipital area, and a full-term infant with an overwhelming enteroviral infection and hepatic necrosis had abnormal accumulation in the lungs and scalp. The etiology of these abnormal accumulations is uncertain; however, bleeding into these areas is a distinct possibility. Although these represent single cases, it is possible that the systematic application of these techniques will improve our understanding of the mechanism of thrombocytopenia in these and other infants.

In this study, the use of ^{111}In -labeled platelets has shown that thrombocytopenia in the newborn infant is primarily caused by increased platelet destruction and, in some patients, platelet sequestration.

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The Effects of Three Recombinant Growth Factors, IL-3, GM-CSF, and G-CSF, on the Blast Cells of Acute Myeloblastic Leukemia Maintained in Short-Term Suspension Culture

By Jun Miyauchi, Colm A. Kelleher, Yu-Chung Yang, Gordon G. Wong, Steven C. Clark, Mark D. Minden, Salomon Minkin, and Ernest A. McCulloch

The blast stem cells of acute myeloblastic leukemia (AML) respond in cell culture to growth factors by both self-renewal and terminal divisions. Both of these functions have been shown to be stimulated by the recombinant growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF). In this paper, recombinant gibbon interleukin-3 (IL-3), homologous to human IL-3, was tested on blast

cells and compared with the effects of GM-CSF, G-CSF, and medium conditioned by the bladder cell line 5637 (5637-CM). We found that IL-3 was an effective stimulator of blast renewal and terminal divisions. However, great patient-to-patient variation was found. A graphic method of presenting complex comparisons between growth factors is also included.

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THE BLAST CELLS of acute myeloblastic leukemia (AML) may be considered as lineage maintained by blast progenitors with the stem cell properties of self-renewal and commitment to terminally dividing end cells that retain blast phenotypic characteristics.¹ Two complementary cell culture methods are applicable to the blast lineage; the first is a clonogenic assay that supports colony formation in methylcellulose by blast progenitors.² Although positive replating experiments have provided evidence for self-renewal during colony formation,³ most of the cells within colonies are proliferatively inert. Self-renewal in culture is more evident in the second method where clonogenic cells increase exponentially in suspension culture.⁴ Both techniques usually require the presence in the cultures of growth factors, which may be supplied by culture supernatants from phytohemagglutinin-stimulated mononuclear cells (PHA-LCM)⁵ or cultures of an active subclone of the continuous bladder carcinoma cell line 5637 (5637-CM).⁶ Alternatively, recombinant growth factors produced by genes encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) or granulocyte colony-stimulating factor (G-CSF) have been shown to be active on blast cells in culture.⁷⁻¹⁰

We have used the two culture assays to show that GM-CSF and G-CSF support both blast self-renewal and terminal divisions, although, as in other biologic characteristics of blast cells, marked patient-to-patient variation was found in growth factor responsiveness.¹⁰ In this paper, the same approach is used in the study of a factor, interleukin-3 (IL-3) (or multi-CSF), that is thought to stimulate early hematopoietic progenitor cells including multipotential progenitor cells.^{11,12} The cDNA encoding the genes for murine IL-3^{13,14} as well as gibbon IL-3¹⁵ has been cloned. An extensive (>99.5%) homology of the human gene with the gibbon sequence has been demonstrated and the gene product, recombinant gibbon IL-3, has also been shown to stimulate both myeloid and erythroid colony formation by human bone marrow cells.¹⁵ We found that this gene product had a major activity in stimulating self-renewal, in addition to some terminal differentiation, of clonogenic cells in AML.

MATERIALS AND METHODS

Cells. Heparinized peripheral blood was obtained at diagnosis or relapse before treatment from five patients with AML (French-American-British [FAB] classification M₂ and M₄) and one patient with blast crisis of chronic myelogenous leukemia. The clinical

information on the patients is shown in Table 1. Normal marrow was obtained, with informed consent, from two donors of marrow for transplantation. These procedures were approved by the Human Subjects Review Committee of the University of Toronto.

T cell-depleted mononuclear cells from peripheral blood and bone marrow were obtained as described previously by using the two-cycle Ficoll-Hypaque procedure, the second after the formation of E-rosettes.⁵ These cells were cultured immediately in suspension⁴ or preserved by freezing in liquid nitrogen in 10% dimethyl sulfoxide and 50% fetal calf serum (FCS).

Hematopoietic growth factors. Recombinant gibbon IL-3 was obtained at the Genetics Institute (GI), Cambridge, MA, as medium conditioned by IL-3-transfected monkey COS-1 cells.¹⁵ Recombinant human GM-CSF was also produced at GI as medium conditioned by Chinese hamster ovary cells.¹⁶ Recombinant human G-CSF was produced at the Ontario Cancer Institute by transfecting COS-1 cells with cloned cDNA for G-CSF that was prepared from TPA30-1, an SV-40-transformed trophoblast cell line, at GI as previously described.¹⁰

Suspension culture. Blast cell populations from fresh samples or cryopreserved stock were suspended in 3 mL of α -minimum essential medium (α -MEM) supplemented with 20% FCS and 10% 5637-CM at a concentration of 10⁶ cells/mL in 35-mm Lux culture dishes (Miles Scientific, Naperville, IL). These cells were used after two to 48 days of suspension culture. Cells were taken from suspension, rather than used directly after T cell depletion or thawing to minimize the possible influences of growth factor carry-over from patients or the influence of co-existing normal cells. For the samples maintained in suspensions, the cells were subcultured every seven days. Fresh normal bone marrow cells were suspended in 20 mL of Iscove's modified Dulbecco's medium (GIBCO, Grand Island, NY)

From the Ontario Cancer Institute, Toronto, and the Genetics Institute, Cambridge, MA.

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Table 1. Cell Populations

Patient	Age (yr)	Peripheral Blood		Diagnosis (FAB)	Time in Suspension (d)
		WBC ($\times 10^9/L$)	Blasts (%)		
1	55	3.3	69	M ₄	32
2	41	118.0	66	*	2
3	64	59.1	36	M ₄	8
4	74	165.0	79	M ₄	48
5	60	3.1	83	M ₂	6
6	65	133.0	95	M ₄	3

*CML, blast crisis.

supplemented with 20% FCS at a concentration of 10^6 cells/mL in a 75-cm² Falcon tissue culture flask (Becton Dickinson, Lincoln Park, NJ). After overnight to two days' incubation, nonadherent cells were harvested and used.

Leukemic blast cells were washed with α -MEM, suspended in 24-mm Linbro multiwell plates (Flow Laboratories, McLean, VA) in 2 mL of α -MEM supplemented with 20% FCS and 10% 5637-CM or CSF (10^{-3} dilution of GM-CSF or IL-3, or 10^{-2} dilution of G-CSF) or without added growth factors at a concentration of 10^6 cells/mL, and cultured at 37°C in a fully humidified atmosphere with 5% CO₂ in air. Seven days later, blast cells were harvested, and the number and morphology of the cells were determined. The cells were then washed with α -MEM and tested for their ability to form blast colonies (PE1). Normal bone marrow cells were cultured in suspension in the same way as for blast cells except that Iscove's medium was used instead of α -MEM. After seven days' suspension culture, the cells were washed with Iscove's medium and used for clonogenic cell assay as described later.

Blast colony assay. Blast colony formation was assessed as previously described.⁵ Every group of cells cultured in suspension was plated in 10-mm Linbro/Titertek microtitration multiwell plates (Flow Laboratories, McLean, VA) at a concentration of 10^4 cells/well in 0.1 mL α -MEM supplemented with 20% FCS, 0.8% methylcellulose, and one of the following growth factors: 10% 5637-CM, 10^{-3} dilution of GM-CSF or IL-3, 10^{-2} dilution of G-CSF, or without any added growth factors. After five to seven days' incubation, colonies consisting of more than 20 cells were counted under an inverted microscope. The yield of clonogenic cells per milliliter was obtained by multiplication of plating efficiency (PE1) by cell recovery. Titration experiments were performed by using the blast colony assay. Blast cells maintained in suspension with 10% 5637-CM were used as target cells for the titration of the three CSFs (IL-3, GM-CSF, and G-CSF) as well as 5637-CM.

Assay for hematopoietic progenitor cells in bone marrow. Bone marrow cells were used for assessment of progenitor cells of all

lineages including multipotential progenitor cells as well as granulocyte and/or macrophage progenitors (CFU-C) by using the techniques described by Messner et al.¹⁷ Cells were plated in 10-mm Linbro/Titertek microtitration multiwell plates at a concentration of 1.5×10^4 cells/well in 0.1 mL Iscove's medium supplemented with 0.8% methylcellulose, 30% fresh human plasma (obtained from a volunteer), 10% PHA-LCM, 5×10^{-5} mol 2-mercaptoethanol, and 0.5 U/mL of recombinant human erythropoietin (Amgen Biologicals, Thousand Oaks, CA). Colonies consisting of more than 50 cells were counted at day 14 of culture.

Morphology. Nonadherent cells harvested from suspension culture were deposited on coverslips with a cytocentrifuge, and adherent cells were washed three times in phosphate-buffered saline. These cell preparations were stained with Giemsa for morphological observation.

RESULTS

Titration of growth factors in methylcellulose culture. The capacity of the recombinant CSFs IL-3, GM-CSF, and G-CSF to stimulate blast colony formation in methylcellulose was titrated by using blast cells cultured in suspension with 10% 5637-CM. As a control, 5637-CM was also titrated by using the same target cells. Figure 1 shows a representative result. Among the three CSFs, GM-CSF was usually the most powerful stimulator of blast colony formation, with a maximum effect at a 10^{-5} dilution. Indeed, GM-CSF was nearly as active as 5637-CM. Stimulation of blast colony formation was also seen with increasing concentrations of IL-3 and G-CSF. Although patient-to-patient variation in the response of blast cells to each CSF was evident, maximum responses were found at dilutions of 10^{-3} for IL-3 and 10^{-2} for G-CSF; these dilutions were used in the subsequent experiments. GM-CSF was tested at 10^{-3} , a concentration usually in excess of that required for maximum effect.

Effects of three recombinant factors on blast renewal and determination. The cells from the six patients were then studied in a detailed experiment designed to compare the effects of IL-3, GM-CSF, and G-CSF on renewal (growth in suspension) and terminal divisions (growth in methylcellulose). The experimental design had two steps. In the first, the cells were cultured in suspension with growth medium and one of the recombinant factors at the concentration stated earlier. As a control, parallel cultures were carried with 5637-CM, the condition under which blast cells are maintained in suspension. The second stage began seven days later when each suspension, including the control in 5637-CM, was collected and the cells were counted, washed, and plated in methylcellulose in the presence of each of the recombinant factors, 5637-CM, or without growth factor. Thus, the first stage shows the effects of the growth factors in suspension and may be considered to reflect their capacity to support blast stem cell renewal. The second stage shows the effects of the growth factors in methylcellulose and may reflect their actions on terminal divisions. By plating each suspension in the recombinant factors, the design also permits the detection of interactions between the growth conditions in suspension and in methylcellulose; if exposure to one factor in suspension (for example IL-3) influenced the growth of clonogenic cells in methylcellulose with the same or different

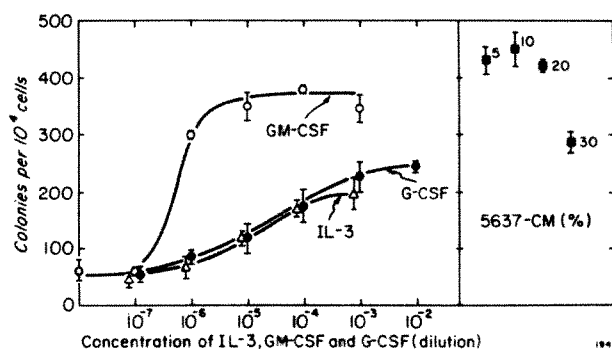


Fig 1. Titration curves of IL-3, GM-CSF, G-CSF, and 5637-CM. Data are from cells of patient 1 that were tested after the cells were maintained in suspension culture for about 2 weeks.

factors, the effect would be evident as a consistent increase or decrease in plating efficiency when the sequence was IL-3 in suspension followed by the putative interacting factor in methylcellulose. This design gives the number of cells after seven days in suspension for the five conditions (Table 2), the plating efficiencies, and the number of clonogenic cells recovered from each suspension (Fig 2).

Cell number. As shown in Table 2, variable increases in cell number were seen in cultures with GM-CSF and G-CSF, and, with the exception of cells from patient 4, the growth was nearly equivalent to that obtained with 5637-CM. IL-3 also increased the cell number in suspension, but it was usually less active than other growth factors; for cells from patient 4, the cell number decreased in cultures with IL-3 but was maintained or increased with the other stimulators. Cells from patients 2 and 5 increased in suspension in the absence of added growth factors; in the latter instance, the addition of factors did not increase growth.

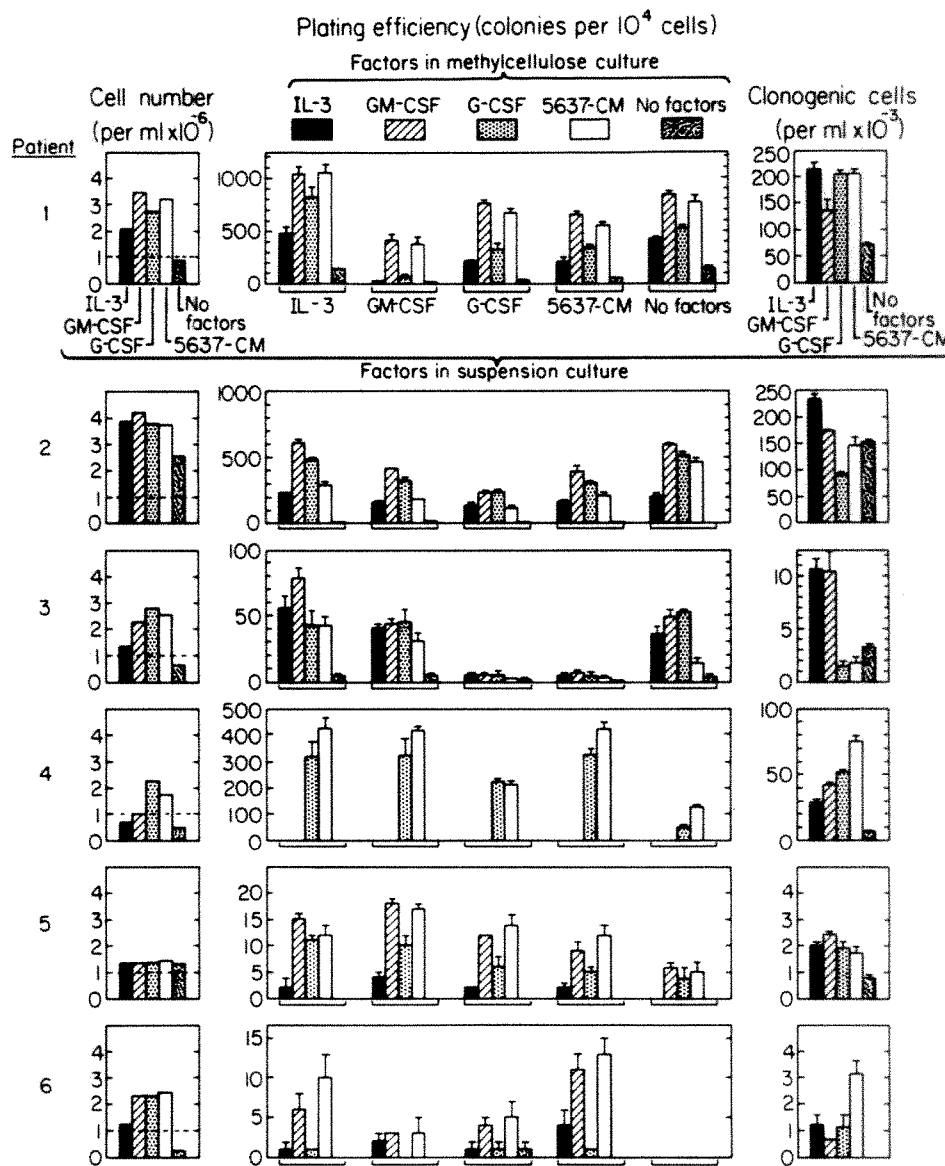
Table 2. Recovery of Cells in Suspension Culture

Patient	Factors in Suspension Culture				
	IL-3	GM-CSF	G-CSF	5637-CM	No Added Factors
1	2.02	3.40	2.70	3.16	0.84
2	3.87	4.20	3.79	3.72	2.56
3	1.35	2.28	2.78	2.58	0.63
4	0.68	1.01	2.31	1.77	0.52
5	1.34	1.35	1.36	1.43	1.32
6	1.23	2.30	2.31	2.44	0.25

The data represent cell numbers $\times 10^{-6}/\text{mL}$ after seven days of suspension culture.

Plating efficiency. Cells from each of the five suspension cultures were plated in methylcellulose with IL-3, GM-CSF, G-CSF, 5637-CM, or no added factors. The data are shown as histograms in Fig 2. For convenience, the cell number data for the five suspension cultures from the cells of each patient

Fig 2. The data for the effects of IL-3, GM-CSF, G-CSF, 5637-CM, and no added factors on AML blasts are shown as histograms. The histograms on the left side are for the cells recovered after seven days in culture (Table 2). The histograms on the right side show the recovery of clonogenic cells obtained from suspensions cultured under different conditions; the highest plating efficiency obtained with any factor in methylcellulose was used to calculate the recovery of clonogenic cells; thus the histograms on the right display the maximum self-renewal that was detected for each of the growth factors to which the cells were exposed in suspension. The histograms in the center of the figure are the plating efficiencies in methylcellulose obtained with each of the five conditions used in suspension. Thus, for example, cells grown in suspension with IL-3 were tested in methylcellulose with IL-3, GM-CSF, G-CSF, 5637-CM, and no added factors. This set of five conditions was used to test cells recovered from each of the suspension cultures in methylcellulose.



(Table 2) are also given as histograms at the left of the figure. The three recombinant growth factors and 5637-CM supported colony formation, although to varying degrees; usually cultures with GM-CSF or G-CSF had the most colonies, and IL-3 was less effective. Cells from patients 2 and 5, independent of factors with respect to an increase in cell number, did not form colonies in methylcellulose without added stimulator. A small background of colony formation was seen in cultures of cells from patients 1 and 3.

Clonogenic cells. The recovery of clonogenic cells in suspension culture was obtained by multiplying the cell recovery (Table 2) by the plating efficiency. The data are given as histograms shown to the right of Fig 2 that depict the maximum clonogenic cell recovery and are based on the greatest plating efficiency achieved with any stimulator. Again, patient-to-patient variation is evident. For blasts from patients 1, 2, and 3, IL-3 was the most potent stimulator of self-renewal, although GM-CSF, G-CSF, and the mixture of factors in 5637-CM were also effective to varying degrees. The cells from patient 4 were stimulated toward self-renewal less effectively by IL-3 than by GM-CSF, G-CSF, and 5637-CM. For cells from patients 5 and 6 the three factors were nearly equivalent.

Test for interactions between factors in suspension and methylcellulose. The plating efficiencies were examined for any consistent pattern that could be related to the growth conditions in suspension. That is, we sought evidence that the exposure of the cells to one growth factor in suspension affected the plating efficiency in methylcellulose in the presence of the same or another factor. The histograms (Fig 2) indicate that, although the combined effect of the growth factor in suspension and in methylcellulose varied from patient to patient, within each patient the effects of the conditions in suspension and methylcellulose appear additive rather than interactive; that is, the contribution to the plating efficiency by the growth factor in suspension is the same for all growth factors in methylcellulose. What the histograms suggest is confirmed by an analysis of variance using the logarithm of plating efficiencies. A separate analysis was done for each patient. Table 3 presents the results of significance tests for the presence of interactions between the condition in suspension and the condition in methylcellulose. The error term is furnished by the replicates. Since the

Table 3. Statistical Analysis for Interactions Between Factors in Suspension and in Methylcellulose

Patient	Factor in Suspension Excluded	Factor in Methylcellulose Excluded	Test for No Interaction (P Value)
1	None	None	.0011
	No GF	No GF	.0005
	No GF & GM-CSF	No GF & GM-CSF	.3205
2	None	None	.0001
	No GF	No GF	.0377
	No GF & G-CSF	No GF	.6012
3	None	None	.0004
	No GF	No GF	.8757
4	None	None	.0816
	No GF	No GF	.1973
5	None	None	.0455
	No GF	No GF	.1648
6	None	None	.0001
	No GF	No GF	.0447
	No GF & GM-CSF	No GF	.3177

Abbreviation: No GF, no growth factor.

absence of growth factors was expected to have a nonspecific and negative impact on colony formation, Table 3 contains the results of the tests for interaction after excluding the conditions where no growth factor was added in either suspension or methylcellulose. For those patients (1, 2, and 6) for whom the interaction remained significant, additional conditions were excluded until the data did not show a significant interaction between the remaining growth factors in suspension and in methylcellulose. For patient 1, it was necessary to exclude GM-CSF in both conditions; omission of GM-CSF in suspension was sufficient for patient 6. G-CSF in suspension had to be left out of the analysis to obtain a nonsignificant *P* value in the case of patient 2. Thus, no consistent effects were seen on the exposure of cells to one growth factor in suspension and the same or a different factor in methylcellulose.

Normal bone marrow cells. Normal marrow cells were tested by using a similar design to that developed for blast populations; cells from two subjects were grown in suspension in IL-3, GM-CSF, or G-CSF and then plated in growth medium with PHA-LCM. The data are summarized in Table 4. All three of the recombinant factors led to increases

Table 4. Summary of Data on Normal Bone Marrow Cells

	Factors in Suspension			No Added Factors
	IL-3	GM-CSF	G-CSF	
Subject 1				
Cell number*	0.89	1.27	1.48	0.45
Plating efficiency†	17 ± 5	5 ± 1	6 ± 1	9 ± 2
CFU-C/mL	1,009 ± 297	423 ± 85	592 ± 99	270 ± 60
Subject 2				
Cell number*	1.61	1.78	3.09	1.17
Plating efficiency†	105 ± 17	48 ± 4	34 ± 8	54 ± 1
CFU-C/mL	11,216 ± 1,811	5,666 ± 533	6,952 ± 1,618	4,173 ± 68

Plating efficiency and CFU-C/mL are shown as means ± SD.

*The data represent cell number × 10⁻⁶/mL after seven days of suspension culture.

†The data represent the number of CFU-C/1.5 × 10⁴ cells plated.

of CFU-C over suspension cultures in growth medium alone. IL-3 was more effective in maintaining or increasing CFU-C than GM-CSF or G-CSF. Colonies in the test of cells from subject 2 were characterized in detail. Cells grown in suspension in IL-3 or GM-CSF yielded more granulocytic than macrophage colonies (89 granulocyte plus 16 macrophage and 44 granulocyte plus four macrophage colonies, respectively). For cultures with G-CSF or no added factors the colonies were nearly equally divided between granulocyte and macrophage compositions. BFU-E colonies were detected in very small numbers in some conditions, but megakaryocyte or mixed colonies were not observed.

Morphology. After seven days of suspension culture, more adherent cells were observed in the presence of GM-CSF than other growth factors, and more numerous monocyte- or macrophage-like cells appeared in the nonadherent cell population of this group.

DISCUSSION

The purpose of the experiments reported in this paper was to compare the effects of three recombinant growth factors on the blast stem cells of AML. The design was based on the view that blast colony formation in viscous medium is largely the result of terminal divisions whereas growth in suspension reflects self-renewal. Support for this interpretation of the two assays has come from experiments with chemotherapeutic drugs. Survival curves were steeper in suspension than in methylcellulose for cytosine arabinoside,¹⁸ a drug that preferentially decreases self-renewal¹⁹; in contrast, 5-azacytidine, which selects for self-renewal events,²⁰ was less toxic when added to suspension cultures than cultures in methylcellulose.²¹ The same approach, used to study recombinant GM-CSF and G-CSF, showed that these, alone or in combination, were active on both blast renewal and determination.¹⁰ In addition, the experiments were designed to permit the detection of effects of exposure to one growth factor in suspension on the subsequent plating efficiencies of the cells grown in methylcellulose with the same or different factors.

Our major finding is that the response of AML blasts to IL-3 shows marked variation from patient to patient; as in the case of the other growth factors,¹⁰ both renewal and determination are stimulated, although to varying degrees for each blast clone. Indeed, the response to growth factors may be considered as an aspect of the phenotype of blast stem cells in each patient. We did not find strong or consistent evidence for interactions between one factor in suspension and others in methylcellulose. Although the results do not exclude such interactions, it seems more plausible to consider that renewal or determination are affected by factors to which they are exposed at a single time and are not influenced by the previous culture history of the blast stem cells. This point is relevant to one aspect of the experimental design generally. Cells were tested after periods ranging from two to 48 days in suspension with 5637-CM. This culture procedure, which in our hands preserves the blast characteristics of the cells,¹⁸ was used so that the pre-test conditions could be common for the six patients studied. The observation that previous conditions may not affect the response of blast populations to growth factors

may have made this precaution unnecessary; moreover, a comparison, in Fig 3, of blast populations 2, 3, 5, and 6 (short times in suspension) to populations 1 and 4 (long times in suspension) does not provide evidence that time in suspension is an attribute contributing to the response to growth factors.

The experimental design used to determine the phenotypes yielded much numerical data; each specimen was cultured in suspension under five conditions, each suspension plated independently in the same conditions, and the total clonogenic cells calculated from each of the 25 plating efficiencies. Thus, 55 numbers were generated for the cells of each patient. We used a graphic representation suggested by Chambers et al²² to display the data. Thus, each value was considered a vector; then, for each growth factor the data for a single population can be shown as an irregular four-sided projection (rhomboid). Then, rhomboids for each growth factor can be superimposed to produce a combined projection characteristic of each patient. The depiction is shown in Fig 3. At the top of the figure, the projection for patient 1 is shown, with the axes of the rhomboid labeled. The top vertex is the cell number after seven days in suspension. The left side is the plating efficiency in methylcellulose with 5637-CM after seven days in suspension with or without recombinant factors. The right side is the result obtained when the cells are maintained in suspension under standard conditions in 5637-CM and plated in methylcellulose with each of the factors or with growth medium alone. The bottom is the calculation of clonogenic cells per milliliter. The depictions of the six patients are shown in the lower part of the figure. However, an interpretation is added. The left and downward direction is considered to reflect an increase in stem cells and therefore "birth," whereas the right-upward direction shows postdetermination events and hence "death" as these terms were used in the stochastic model of stem cell regulation.²³ Each growth factor is color coded.

Examination of the projections emphasizes the patient-to-patient variation; it is also possible to estimate the responsiveness to growth factors since this parameter is related, although not directly, to the area of each projection. The balance between "birth" and "death" can be deduced from the amount of each projection to the left and down as compared to the right and up. For example, the projection for patient 2's cells shows a large green rhomboid extending to the left and illustrating self-renewal or maintenance of clonogenic cells in the absence of added factors. Each of the recombinants moves the projection to the right, although IL-3 does so less than the others, which indicates that, for this instance, the balance is shifted by growth factors towards determination. In contrast, the rhomboids for patient 6's cells are small and show poor growth in culture; IL-3 moves the population toward renewal, and GM-CSF favors determination. The reader may make similar deductions for the other samples from consideration of the projections.

For comparison with the blast cell data, a similar experimental design was applied to normal marrow from two individuals. In both, IL-3 was the most active factor in stimulating an increase in CFU-C in suspension culture.

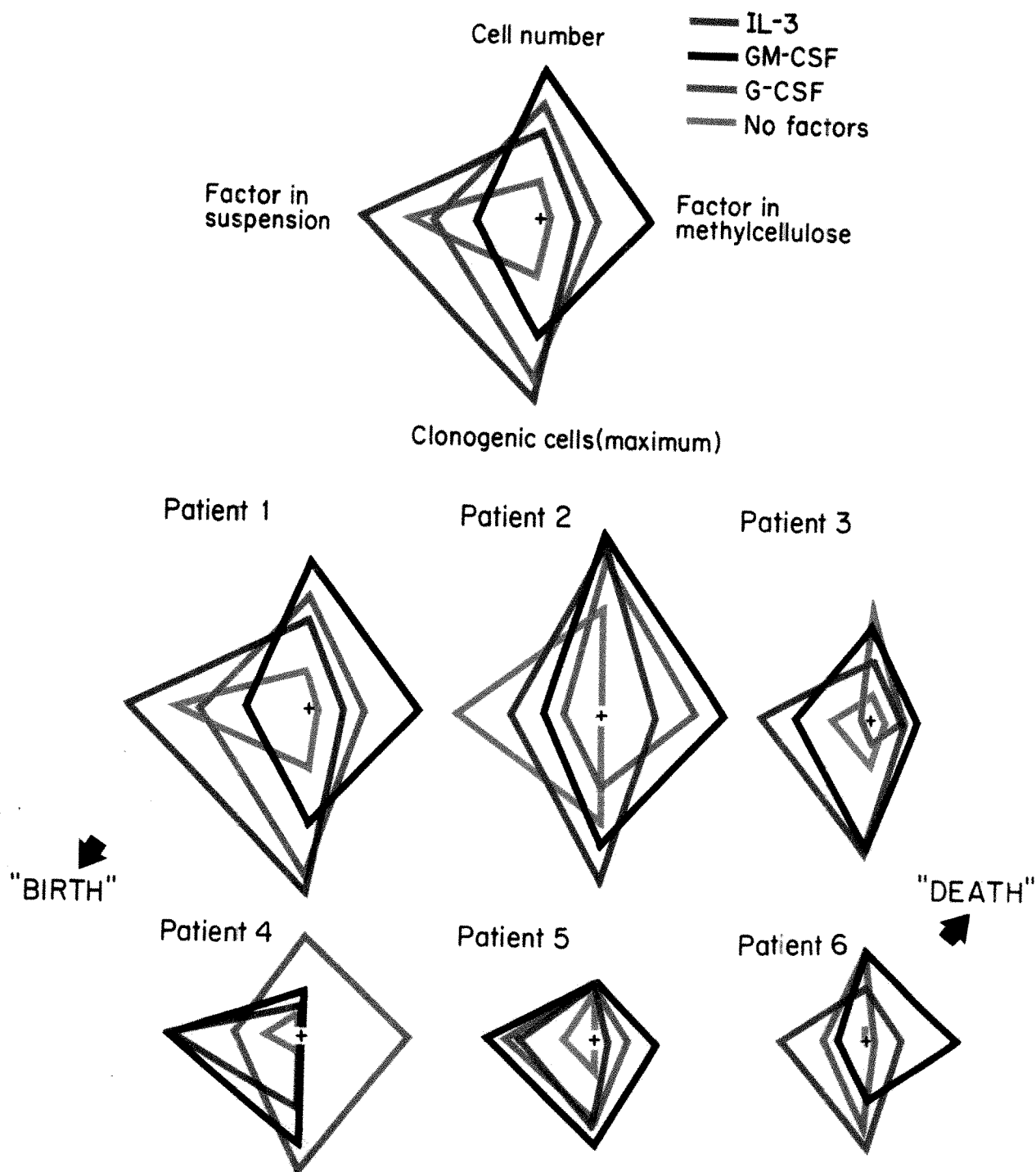


Fig 3. A graphic representation of the combined data for the six patients that uses a representational method devised from those described by Chambers et al.²² Lines drawn to scale are used to show cell recovery in suspension (upper direction), maximum recovery of clonogenic cells after growth in suspension (lower direction), 5637-CM-stimulated plating efficiency after seven days in suspension under each of culture conditions (line to the left), and the standard plating efficiency obtained from cells grown in 5637-CM and plated in each CSF (line to the right). The ends of the lines are joined to form rhomboids, one for each growth factor. The scales are labeled in the figure so as to represent the data for patient 1's cells at the upper part of the figure. Similar projections are given for the six patients in the lower part of the figure. However, directions down and to the left are labeled "birth" since the responses symbolized by the lines to the left and down indicate self-renewal. In contrast, directions to the right and up are labeled "death" since responses symbolized by the lines to the right and up reflect terminal divisions. The lines are scaled comparably for data from each patient. However, because of the marked patient-to-patient variation, the scales could not be uniform; for samples with low plating efficiencies (for example, those from patients 5 and 6) the scales were increased. Thus, the areas of the rhomboids are accurate representations of the magnitude of each effect within the diagram for each patient, but the patient-to-patient variation is approximate and underestimated. For an interpretation, see the text.

Unlike blast suspension cultures where increases in clonogenic cells may be attributed to self-renewal, CFU-C in suspension may be generated by recruitment from more primitive precursors.²⁴ Our findings on normal marrow, therefore, support the view that IL-3 acts on early marrow stem cells.

We consider it important to depict the phenotypes of blast populations for two reasons. First, the phenotype of each patient may contribute to the outcome of treatment. Second, and perhaps of greater importance, the phenotype should be considered before any factor is given in vivo.

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Immunologic Reconstitution After Haploidentical Bone Marrow Transplantation for Immune Deficiency Disorders: Treatment of Bone Marrow Cells With Monoclonal Antibody CT-2 and Complement

By R.C. Moen, S.D. Horowitz, P.M. Sondel, W.R. Borchering, M.E. Trigg, R. Billing, and R. Hong

Patients with congenital T lymphocyte deficiency disorders received transplants with parental bone marrow depleted of mature T cells by the use of an anti-T cell monoclonal antibody (CT-2) and complement. Our results with 16 consecutive patients (20 transplants) showed rapid engraftment of donor cells; cytoreduction (busulfan, cytosine arabinoside [ara-C], cyclophosphamide) was used in six transplants, and marrow ablation was used in six (ara-C, cyclophosphamide, 1,365-cGy total body irradiation). No patient received prophylactic anti-graft-v-host disease (GVHD) therapy posttransplant, and only one patient developed significant GVHD, which involved the skin, liver, and gastrointestinal tract. Seven others showed some manifestations of GVHD, but these were of minimal clinical significance and required only occasional steroid

therapy. Overall, eight patients are alive and well; eight did not survive. Polyclonal immunoglobulin synthesis by donor memory B cells was seen shortly after transplantation, with peak donor-derived serum levels seen approximately 2 months after transplantation. After this initial immunoglobulin synthesis waned, another wave of B cell responses developed. This immunoglobulin response appears to be permanent. T cell functions appeared as soon as 3 weeks after transplantation. This experience in a variety of patients with combined immunodeficiency who received transplants with monoclonal antibody T cell-depleted marrow shows gratifying results with a consistent T and B cell benefit.

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SEVERE COMBINED IMMUNE DEFICIENCY (SCID) can be successfully treated with transplantation of bone marrow from HLA-identical siblings. Unfortunately, the majority of affected individuals will lack an HLA-identical bone marrow transplant (BMT) donor. To offer BMT therapy to patients without suitably matched stem cell donors, immunocompetent T cells have been removed from bone marrow by antibodies to T cells¹⁻⁴ or by agglutination with soybean lectin or sheep erythrocytes.⁵⁻⁸ We report our results in 16 consecutive patients with immunodeficiency syndromes receiving parental marrow depleted of T lymphocytes by the use of the monoclonal antibody CT-2 and complement. This procedure has resulted in marrow engraftment and reconstitution of both B and T cell functions with minimal graft-v-host disease (GVHD).

MATERIALS AND METHODS

Donor and recipient characteristics. The diagnoses of the patients included SCID (adenosine deaminase [ADA]-positive and -negative), Wiskott-Aldrich syndrome (WAS), and cartilage-hair hypoplasia (CHH) (Tables 1 and 2). Of the 11 ADA-positive SCID

patients, eight had disproportionate amounts of B cells, the most common variety. Three patients had normal to elevated Ig responses but had had prior thymus transplants. Three (patient nos. 34, 86, and 87) had, before therapy, levels of immunoglobulins consistent with the diagnosis of SCID with immunoglobulins. Informed written consent for bone marrow transplantation with donor bone marrow depleted of T lymphocytes was obtained. All protocols for transplantation were approved by the Human Subjects Committee of the University of Wisconsin.

BMT preparation regimens. Busulfan (4 mg/kg/d orally for four days) was used for patients under 2 years of age to avoid the CNS toxicity of total body irradiation (TBI) (Table 1). Otherwise, fractionated TBI was administered at a dose of 1,320 cGy (165 cGy $\times$ 8) with only the eyes shielded (patient no. 80) or both eyes and lungs shielded (patient nos. 87 and 100). Cyclophosphamide was used in all patients receiving myeloablative therapy. Cytosine arabinoside (six doses of 3 g/m² or 2 g/m² [transplant 105]) was added to the preparative regimen to further immunosuppress the recipient because the experience with patient no. 26 and with patients receiving T cell-depleted BMT for hematologic malignancy has demonstrated that further immunosuppression is necessary to prevent graft rejection by the host.⁹

Bone marrow harvest, T cell depletion, and transplantation. Bone marrow was aspirated from posterior iliac crests, depleted of T cells, and infused as described previously.⁴ Only one cycle of antibody and complement were used for each marrow treatment. E rosette-positive and T11-positive cells are completely removed by this treatment (T11+ cells were measured by flow cytometry). Mixed leukocyte reactivity and cytotoxic T cell generation against allogeneic targets was usually but not invariably decreased. Patient nos. 28 to 115 also received a portion (5% to 30%) of donor marrow cells directly into their marrow space. Four to 15 mL of concentrated marrow cells was injected into the posterior iliac crest(s).¹⁰

Assessment of engraftment and GVHD. The latest assessment of T lymphocytes is shown in Table 3. Engraftment of donor marrow was also documented by HLA phenotyping of peripheral blood lymphocytes and where possible by karyotypic analysis of peripheral blood lymphocytes and RBC phenotyping.

The severity of GVHD was defined by the Seattle criteria.¹¹ Diarrhea fluid loss for staging of GVHD of the gastrointestinal tract was adjusted according to patient surface area, assuming an average adult value of 1.73.²

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Table 1. Clinical Data

UPN*	Disease	Age†	Prep‡	Prev Tx§	State of Health	BM Dose¶	GVHD# (S/L/G)	Months F/U**	Outcome††
Alive									
028	SCID	20	None	CTF	Good	11.3	2/1/0	39	Well
057	SCID	6	None	None	Fair	12.2	1/0/0	27	Well
080	SCID	55	ARA-6, C2, TBI	CTF	Good	4.7	1/0/0	19	Well
087	SCID	65	ARA-6, C2, TBI	TP5	Good	4.6	1/0/0	16	Well
094	SCID	10	None	None	Good	19.4	3/0/0	15	Well
026	WAS	2	B4, C4	None	Good	9.8	0/0/0	39	Well
100a	ADA	83	None	CTF	Poor	4.8	0/0/0	2.5	Temporary engr
100b		85	ARA-6, C2, TBI	None	Poor	3.7	1/2/0	10	Improved
105	ADA	4	ARA-6, C2, B4	None	Fair	5.7	1/0/0	14	Well
Not alive									
023a	SCID	12	None	None	Good	8.1	0/0/0	5	No engraftment
023b		17	B4, C4	See 23a	Poor	8.4	NE	—	Died (4)
027	SCID	56	None	CTF	Poor	9.5	2/1/0	—	Died (28)
034	SCID	52	B4, C4	None	Good	5.0	NE	—	Died (0)
086a	SCID	23	None	None	Fair	6.5	0/0/0	9	Temporary engr
086b		34	ARA-6, C2, TBI	See 86a	Poor		NE	—	Died (—2)
093	SCID	58	ARA-6, C2, TBI	CTF	Poor	6.6	NE	—	Died (14)
099a	SCID	80	None	CTF	Good	9.6	0/0/0	5	No engraftment
099b		86	ARA-6, C2, TBI	See 99a	Good	8.0	2/1/0	—	Died (22)
110	CHH	13	ARA-6, C2, B4	None	Fair	6.1	3/2/2	—	Died (15)
115	ADA	27	ARA-6, C2, B4	None	Good	12.8	1/0/0	—	Died (9)

*UPN, unique patient number. Patient nos. 23, 86, 99, and 100 received transplants on two occasions (a = 1st transplant; b = 2nd transplant). Only patient nos. 87 and 100 are female.

†Age at the time of transplant in months.

‡B4, busulfan, 4 mg/kg/d × 4; C4, Cytosan, 50 mg/kg/d × 4; C2, Cytosan, 50 mg/kg/d × 2 except for patient nos. 105, 110, and 115 (45 mg/kg/d × 2); ARA-6, ara C, 3 g/m² every 12 hours × 6 except patient nos. 105, 110, and 115 where the dose was 2 g/m²; TBI, 1,320 rad in eight divided doses. Patient no. 7 had his eyes shielded. Patient nos. 87 and 100 had lungs and eyes shielded.

§Previous therapy: CTF, cultured thymus fragment; TP5, thymopoietin pentapeptide.

||State of health at the time of transplant: good, essentially normal; fair, slightly undernourished, past history of serious infection; poor, actively infected, marked wasting, often chronic diarrhea.

¶Dose of nucleated cells × 10⁸/kg body weight administered after T cell depletion.

#S/L/G indicates the degree of involvement of skin, liver, and gut, respectively. The number indicates the clinical stage of involvement using Seattle criteria (see the text). NE, not evaluated.

**Months of follow-up since transplant.

††See the text for details of death. Immune status is shown in subsequent tables. Temporary engr, temporary engraftment. The numbers in parentheses are the days of death relative to BMT.

Monoclonal antibody cell surface markers. Antibodies directed against various mononuclear cell populations were used for flow cytometric analysis. The antibodies used were OKT3, OKT4, OKT6, OKT8, Leu 11, Leu 7, anti-HLA-DR, B1, and B4.

T cell functions. Mononuclear cells obtained by Ficoll-Hypaque separation were assessed for proliferative responses to mitogens, antigens and alloantigens and cytotoxic responses to alloantigen targets by the usual methods.¹²

B cell function testing. Serum immunoglobulin levels were determined by rate nephelometry using a Beckman ICS Analyzer II (Beckman Instruments, Inc, Fullerton, CA). Specific antibody levels of IgG, IgA, and IgM to the antigens *Escherichia coli*, tetanus toxoid, and diphtheria-tetanus toxoids were determined by an enzyme-linked immunosorbent assay (ELISA).¹³ IgE levels were determined by using an ELISA assay (Epsilon IgE, Beckman Instruments, Inc, Carlsbad, CA).

RESULTS

As shown in Table 1, 16 patients overall received transplants. Eight are alive and well; eight have died. The major cause of death was infection. Of the 16 transplant recipients there were 11 patients with ADA-positive SCID, three

patients with ADA deficiency, one with CHH, and one patient with WAS. The age at the time of transplant varied from 2 months to 7²/₁₂ years. The father was the BMT donor for all transplants except for patient 99 where the mother served as the donor. All donors and recipients were haplo-identical, except as noted. Donor cells were reactive against the host in mixed lymphocyte culture (MLC) except for patient no. 26 who was MLC compatible, HLA-B, -C, and -DR identical, but HLA-A disparate with his donor (Table 2). Cyto reduction or ablative pretreatment was used in 12 of the 20 transplants performed. At the time of transplant or transplant conditioning, the recipient was in good health in ten, fair health in four (transplant nos. 57, 86, 105, and 110), and in poor health in six (transplant nos. 23, 27, 86, 93, and 100) instances.

The number of treated nucleated bone marrow cells received varied from 3.7 × 10⁸/kg to 19.4 × 10⁸/kg. In those patients receiving immunosuppressive treatment before transplantation, engraftment of WBC was present 12 to 20 days after transplantation. Patients nos. 23 and 99 did not show engraftment and died during a second transplant

Table 2. Immunologic Characteristics at Time of Transplant

UPN	Lymphs*	T11	T3	T4	T8	MLC†	B1	IgG	IgA	IgM	HLA Dispar‡	D v R MLC§
Alive												
SCID												
028	273	1	1	0	0	0	15	324	<4	16	2	+
057	2,000	0	0	0	0	0	34	62	<7	30	2	8
080	1,000	14	15	0	5	+	18	736	<7	71	2	+
087	600	6	6	3	2	0	17	464¶	42	95	2	62
094	700	13	5	1	4	0	74	180	<7	41	2	+
WAS												
026	5,000	23	ND	7	12	+	7	778	14	21	1	0
ADA deficiency												
100	80	8	20	6	5	+	4	1,140	114	71	2	+
105	5,000	75	13	11	21	ND	6	160	<6	<6	2	22
Not Alive												
SCID												
023	6,000	55	1	0	3	0	0	138	<3	11	2	+
027	1,392	ND	8	6	5	0	30	272	13	35	2	+
034	924	ND	30	4	21	+	0	460¶	10	35	1	+
086	500	23	4	4	13	+	61	950¶	26	246	2	+
093	300	62	59	4	58	0	6	3,489#	4	457	2	122
099	300	65	17	13	16	0	13	15	3	5	2	+
CHH												
110	1,500	26	10	5	12	+	29	1,170	221	220	2	63
ADA deficiency												
115	360	43	38	20	22	0	6	414	45	20	2	+

*Total number of lymphocytes per microliter.

†O, proliferative value below normal range; +, normal range (stimulation index of 24 to 313); ND, not determined.

‡Degree of HLA disparity. All recipients and donors matched at least one haplotype. The number shown is the disparity for the HLA-A and -B loci of the unmatched haplotype. All patients except no. 026 were mismatched at both loci. The donor and recipient in this case matched at HLA-A but were disparate at HLA-B.

§MLC reactivity of the donor when stimulated by the recipient's irradiated cells. The number is a relative response where the reactivity of the donor v the recipient is divided by the reactivity v a third party and expressed as a percentage. Where a third-party response was not measured, a positive response against the recipient (>5 × stimulation index) is shown as +. All donors showed reactivity in the normal range of proliferation (+) except for patient no. 026 who is MLC identical.

||Previous thymus transplant.

¶SCID with immunoglobulins.

#Previous thymus transplant, stage IV Hodgkin's disease.

Table 3. T Cell Follow-up

UPN	Months* F/U	Lymphocyte Count (× 10 ⁻³ /μL)	OKT3† (%)	OKT4/OKT8	PHA‡ (× 10 ⁻³ cpm)	Con A§ (× 10 ⁻³ cpm)	PWM (× 10 ⁻³ cpm)	MLC¶ (× 10 ⁻³ cpm)
028	39	3.2	69	39/28	304	136	50	62
057	27	2.8	70	46/22	170	53	57	91
080	19	2.1	45	31/17	289	194	48	104
087	16	2.2	63	15/47	47	44	20	54
094	15	3.8	53	32/18	348	212	72	60
026	39	2.6	60	20/35	172	5	10	166
100b	10	0.6	33	20/15	15	12	6	13
105	14	2.0	59	37/18	68	15	19	NT#

*Months follow-up since transplantation.

†Percentage of total lymphocytes.

‡Normal range for PHA response: 51,000 to 281,000 cpm.

§Normal range for Con A response: 16,000 to 224,000 cpm.

||Normal range for PWM response: 5,000 to 99,000 cpm.

¶Normal range for MLC response: 8,000 to 101,000 cpm.

#Not tested.

attempt. In transplant nos. 86 and 100 only transient engraftment occurred; patient no. 86 died before a retransplant could be performed, but patient no. 100 has subsequently received a successful transplant. Thus, in toto, of the 16 patients, 8 are alive and well and show engraftment. Patient no. 26 initially showed donor engraftment of all cell lines. He has continued to demonstrate good donor T cell engraftment, but his red cells, platelets, monocytes, and B lymphocytes are now entirely of the host type. He is chimeric for T cells.

GVHD. Engraftment occurred without significant GVHD (Table 1). Only one patient (no. 110) demonstrated GVHD of greater than grade 2 severity. In patient no. 94 stage III skin GVHD was present without liver or gastrointestinal involvement. This patient and patient no. 110 were treated with high-dose steroid therapy to control the acute GVHD.¹⁴ Patient nos. 28, 57, 87, and 100 required low-dose prednisone therapy (1 to 2 mg/kg) for brief periods of time (1 to 3 weeks). Patient nos. 80 and 105 did not receive any steroid therapy for transient acute GVHD of the skin with minimal liver involvement. Only patient no. 110 had clinical evidence of gastrointestinal GVHD. Chronic GVHD has not occurred.

T cell reconstitution. From 3 to 12 weeks were required before greater than 100 cells/ μ L stained with each of the monoclonal antibodies listed. At no time after transplantation were cells staining with monoclonal antibody OKT6 (indicative of thymocytes) found in peripheral blood. In all cases, the number of T8+ cells exceeded the number of T4+ cells for many months after transplantation. Table 3 lists the relative number of T cells seen at the time of the most recent follow-up.

Six to 18 weeks were required after transplantation before significant proliferation was observed in response to the mitogens phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM) and 48 weeks for the soluble antigen *Candida*. Significant proliferation was defined as the attainment of a level of proliferation in excess of 3 SD of the mean response observed before treatment in 34 newly diagnosed patients with severe combined immunodeficiency syndrome (ADA+). Allogeneic cell (MLC) reactivity was found by the 12th week. In almost all cases (except patient no. 100) the proliferative responses were preceded by the acquisition of significant numbers of T cells as determined by monoclonal markers.

B cell reconstitution. The acquisition of B cell function revealed a novel pattern. Since all patients were receiving intravenous gamma globulin preparations, we evaluated endogenous B cell function by following the IgA and IgM responses. Most patients showed substantial elevations in IgA and/or IgM levels, which peaked about 2 months after transplantation. The serum levels subsequently decreased only to rise again many months later. These immunoglobulin changes occurred in all patients, even those not requiring any blood product support posttransplant. Table 4 provides a summary of current immunoglobulin levels and functional antibody analyses for all surviving patients.

The IgE level remained <50 IU/mL in all of our patients except patient nos. 80 and 100. Patient no. 80 had a peak IgE

level of 3,044 IU/mL 60 days after transplantation. Patient no. 100 had a peak IgE level of 141 at 3 months after receiving her second transplant. The IgE level does not relate to the extent of GVHD seen in our patients. Table 4 lists the current levels of IgE seen in our patients.

DISCUSSION

From February 1983 through September 1985, 16 patients received a total of 20 BMT from haploidentical parents. Of the 16 recipients, eight survived and are living at home in an unprotected environment. All of the children of school age attend public school. Failures were primarily due to infection that was usually present at the time of transplantation. One patient died of cardiotoxicity attributed to cyclophosphamide.

A major decision in bone marrow transplantation for primary immunodeficiency states involves determining the need for ablation. From the published experience, pretransplant conditioning is clearly indicated in ADA deficiency^{8,15} and WAS.^{15,16} In our experience, we have been unable to achieve engraftment even with genotypically identical donor-recipient pairs when the recipient has a normal MLC response (three cases) or in two cases of short-limbed dwarfism (unpublished data). Because of the known heterogeneity of the combined immunodeficiency syndromes, it is extremely important to document all cases of nonengraftment in well-defined immunodeficiency syndromes so that suitable criteria can eventually be developed.

We did not test for silent engraftment of maternal cells, which could affect graft acceptance and may affect B cell reconstitution.¹⁷ However, in those patients who survived, the B cell reconstitution has not been significantly compromised. The role of silent engraftment in the complex biology of bone marrow transplantation needs further definition.

The decision to pretreat is also contingent upon whether a genotypically identical (MLC-nonreactive, HLA-identical sibling) donor or a haploidentical donor is used. Children with ADA deficiency have successfully received grafts with genotypically identical BMT without prior cytoreduction (O'Reilly¹⁵ and personal observations). However, recent unsuccessful attempts with lectin-depleted marrow obtained from haploidentical donors clearly demonstrate the need for prior chemotherapy in ADA deficiency.⁸ Previous unsuccessful attempts at engraftment on patients with hematological malignancy by using T cell-depleted haploidentical marrow donors after conventional ablative regimens show a need for additional immunosuppression to achieve engraftment.⁹ This principle must be extended to primary immunodeficiency states as well.

The optimal cytoreduction or ablation regimen remains to be determined. Although busulfan-cyclophosphamide cytoreduction was shown to be adequate in 14 of 15 transplants in WAS when a genotypically identical donor was used,¹⁵ our results with patient no. 2 suggest that this regimen was inadequate. Similar results have been reported by others when the donor was not genotypically identical.¹⁸ On the basis of the previous experience with monoclonal antibody T cell-depleted marrow transplantation,⁹ we have added cytosine arabinoside to the busulfan-cyclophosphamide regimen.

Table 4. B Cell Assessment

UPN	Ig	Responses to Specific Antigens*						Total Ig†	
		E coli		Diphtheria		Tetanus		Isotypes	IgE
		Before‡	Now‡	Before	Now	Before	Now		
028	IgG	0	150	0	120	0	42	622	<10
	IgA	0	2	0	1	0	3	<7	
	IgM	0	70	0	64	0	4	81	
057	IgG	0	11	0	3	0	26	336	<10
	IgA	0	9	0	3	0	11	31	
	IgM	0	14	0	89	0	20	23	
080	IgG	0	72	0	46	0	31	653	160
	IgA	0	11	0	10	0	4	16	
	IgM	2	34	2	137	2	41	310	
087	IgG	0	36	0	30	0	22	884	<10
	IgA	0	65	0	16	0	32	2,340	
	IgM	0	61	0	57	0	14	298	
094	IgG	0	68	0	84	0	60	530	12
	IgA	0	30	0	7	0	4	13	
	IgM	0	26	0	19	0	17	44	
026	IgG	0	52	0	109	0	59	728	26
	IgA	0	17	0	48	0	18	62	
	IgM	0	76	0	126	0	79	62	
100b	IgG	0	60§	0	36§	0	72§	857	141
	IgA	0	0	0	0	0	0	8	
	IgM	0	0	0	0	0	0	171	
105	IgG	ND		<.01	>6.0	.08	1.2	1,260	NT
	IgA							52	
	IgM							162	

*Results are expressed as a percentage of normal (except patient 105). Values in excess of 5% are within normal range; no patient except 100b received IgG therapy.

†Total isotype levels: IgG, IgA, and IgM are in the left column; IgE is on the right.

‡Assay before transplantation (before) and the latest value (now).

§IgG values attributed to intravenous IgG therapy.

||Values are expressed as units. A tenfold rise is normal.

However, whether this degree of immunosuppression is necessary for recipients with the weaker immune response of SCID is at present unknown. We use a regimen modified from our program in transplantation for patients with hematologic malignancy. Because of the possibility of cardiotoxicity as exemplified by patient no. 115, determination of a minimal dosage is necessary.¹⁹

There was evidence for B cell antibody synthesis of donor memory B cell origin. Antibodies to diphtheria and tetanus toxoids were found in patient nos. 24 and 57 although they had never been immunized. These antitoxin antibodies decrease in titer after day 60 posttransplant. After immunization of the recipients, the titers rose.

Other donor memory B cell antibody synthesis was seen in those instances where the donor and recipient differed at ABO blood groups: patient nos. 57, 87, and 105 (donor, O; recipient, A); patient no. 26 (donor, B; recipient, A); and patient no. 100 (donor, B; recipient, AB). In all patients except patients nos. 26 and 105, the recipient developed hemolysis ten to 16 days after transplantation, and in these patients the serum was shown to contain significant titers of antibodies to host blood groups. Production of donor antihist erythrocyte antibody has been observed by others.^{20,21}

Although two of the patients show definite IgA deficiency and two more may be showing decreasing levels, there is no

evidence that clinically significant disease can be ascribed to this fault. All patients are living at home in an unprotected environment, and no unusual infectious episodes have occurred. Intravenous IgG therapy has been stopped in all patients except patient no. 100. Continued immunoglobulin support has been required after other T cell-depleted bone marrow transplants from haploidentical donors.^{8,22}

Many of the patients had easily detectable and sometimes normal numbers of circulating B cells before transplantation. Histocompatibility testing suggests that all of the lymphocytes are of donor origin except in case no. 27 (WAS-T cell chimera). However, a small number of residual host B lymphocytes might not be detected by usual tissue typing techniques. Studies to directly assess the source of the functioning B cells are in progress.

Although B cell lymphoma has been observed in some instances of T cell-depleted haploidentical marrow administration, no cases occurred in this series. In our center, this complication has not been seen since 1984, at which time routine acyclovir administration post-BMT was introduced.²³

This report shows that T cell-depleted haploidentical marrow obtained by monoclonal antibody and rabbit complement purging can be administered with minimal risk of GVHD and excellent B cell as well as T cell reconstitution

consistently observed in patients with primary immunodeficiency. Cytoablation or ablation is often necessary but needs to be properly defined and the agents carefully chosen so as not to significantly add to the morbidity of the transplant procedure.

NOTE ADDED IN PROOF

Patient no. 100 now has normal immunity. Patient no. 26 was retransplanted without further immunosuppression or T cell depletion of the marrow and has a normal platelet count.

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Induction of Proliferation of B Prolymphocytic Leukemia Cells by Phorbol Ester and Native or Recombinant Interferon- γ

By R.W. Sauerwein, W.G.J. van der Meer, and L.A. Aarden

Phorbol ester phorbol myristate acetate (PMA) induces proliferation in nonmalignant human B cells and B cells from a patient with B prolymphocytic leukemia (B-PLL). Mitogen-free T cell-derived conditioned medium acts synergistically with PMA in inducing proliferation of B-PLL cells but does not enhance the PMA-stimulated outgrowth of nonmalignant B cells. Interleukin 2 (IL-2) has no effect on the outgrowth of B-PLL cells, and monoclonal antibodies against the IL-2 receptor do not influence the response to PMA and conditioned medium. Recombinant interferon- γ (IFN- γ), in contrast, is a potent enhancer of PMA-induced

proliferation of B-PLL cells. With gel filtration techniques and with the use of anti-IFN- γ antibodies, it is shown that IFN- γ in the conditioned medium is responsible for the observed increase in B-PLL cell proliferation. Preincubation of B-PLL cells with IFN- γ induces responsiveness to PMA, whereas IFN- γ alone had no effect on these cells when pretreated with PMA. The combined data show that, in the presence of PMA, native and recombinant IFN- γ are growth factors for B cells from a B-PLL patient and that IL-2 is not involved in this process.

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THE SUPERNATANT OF mitogen- or antigen-activated T cells contains factors with the capacity to regulate B-cell proliferation *in vitro*.¹ Not only normal human B cells but also malignant B cells, which are considered to be arrested in a particular stage of B cell ontogeny, can be induced to proliferate upon the addition of T cell-derived factor(s).

Two genetically well defined lymphokines are usually present in such preparations, ie, interleukin 2 (IL-2)² and interferon- γ (IFN- γ).³ Both factors can act on the proliferation of normal and neoplastic B cells. IL-2 stimulates the outgrowth of normal preactivated human B cells,^{4,5} Epstein-Barr virus-transformed cells,⁶ and prestimulated B cells from patients with B chronic lymphocytic leukemia (B-CLL).⁷⁻⁹ The role of IFN- γ in B cell proliferation is not very well established. IFN- γ does not affect the outgrowth of normal human B cells,¹⁰ whereas in other studies it costimulates proliferation of anti- μ -activated B cells.^{11,12} Unstimulated B-CLL cells have been shown to proliferate in response to IFN- γ .¹³ In murine systems, proliferation of B cells is either inhibited or not influenced by IFN- γ , depending on the costimulatory conditions for these cells.¹⁴

Recently, we showed that B cells from five out of five patients with B prolymphocytic leukemia (B-PLL) proliferated *in vitro* when incubated with the phorbol ester phorbol myristate acetate (PMA). Upon the addition of T cell-derived conditioned medium, PMA-stimulated B cells of one patient showed a synergistic enhancement in proliferation.^{14a}

The proliferative effect of PMA on human B cells has been previously described.^{1,7,15} The aim of the present study was to investigate the nature of the factor in this preparation that was responsible for the increased proliferation. Our data indicate that IFN- γ and not IL-2 is a potent stimulator of B-PLL cell outgrowth and that IFN- γ is at least partially responsible for the proliferation induced by this T cell supernatant.

MATERIALS AND METHODS

Cell Preparations and Separations

B-PLL cells. Isolation of B cells from B-PLL patient W.V. has been described elsewhere.^{14a} Briefly, mononuclear cells (MNC) from splenic origin were isolated by Percoll density gradient centrifugation (1.078 g/mL) (Pharmacia Fine Chemicals, Uppsala, Sweden). This cell suspension contained 95% B1⁺, μ ⁺, δ ⁺, γ ⁺, α ⁺ cells and 4% T3-positive cells. B-PLL cells were depleted of residual T cells by two cycles of E rosette sedimentation with sheep erythrocytes treated with 2-aminoethylisothiuronium bromide hydrobromide (AET).¹⁶

Nonmalignant MNC, B, and T cells. Tonsil fragments that were obtained from either adults or children undergoing tonsillectomy were minced and passed through stainless steel mesh in Earle's medium. Mononuclear tonsil cells were obtained by Percoll density gradient centrifugation as described earlier. Non-T cells and T cells were separated by E rosette sedimentation with AET-treated sheep erythrocytes.¹⁶ This procedure was repeated twice. T cells were obtained by sheep erythrocyte lysis with 155 mmol/L buffered NH₄Cl for five minutes at 0°C followed by three washing steps. Non-T cells were further enriched for B cells: 10⁷ non-T cells/mL in Earle's medium were incubated for 30 minutes on ice with a mixture of anti-Leu-1 (0.25 μ g/mL), anti-Leu-M3 (1 μ g/mL) and anti-Leu-11b (1 μ g/mL) (Becton Dickinson, Sunnyvale, CA). Cells were washed and gently rocked in Earle's medium with 30% (vol/vol) rabbit serum as a source of complement for 45 minutes at 37°C. The cells were washed at least four times before culture and contained more than 95% B1⁺, μ ⁺ cells and less than 0.5% T3-positive cells.

Production and Partial Purification of Conditioned Medium

Mononuclear tonsil cells (10⁶/mL) and cells from a lymphoblastoid cell line CLB TT (3,000-rad irradiated; 10⁵ cells/mL) as stimulator cells were cocultured in culture flasks (Nunc, Roskilde, Denmark) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2% (vol/vol) fetal calf serum (FCS; GIBCO, Grand Island, NY). After 48 hours, the supernatant was harvested, passed over a 0.22- μ m Millipore filter (Millipore Corp, Freehold, NJ), and

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stored (-20°C) until used. This material will be referred to as MLC-CM. MLC-CM was concentrated 30 times by ultrafiltration over an Amicon YM-10 filter (Amicon Corp, Danvers, MA) and fractionated by gel filtration on an AcA54 column (LKB, Bromma, Sweden) in a buffer with 0.15 mol/L NaCl, 100 μg polyethyleneglycol (PEG) 4000/mL, and 5 mmol/L HEPES, pH 7.2.

Preparation and Purification of IL-2

Cells (10^6 /mL) from the human leukemic T cell line Jurkat were cultured in IMDM in the absence of proteins with 100 μg /mL PEG 4000 and stimulated with phytohemagglutinin (1 μg /mL) and PMA (10 ng/mL). After 24 hours the supernatant was harvested, concentrated, and passed over an AcA54 column (LKB) in a buffer with 0.15 mol/L NaCl, 100 μg PEG 4000/mL and 5 mmol/L HEPES, pH 7.2. The IL-2-containing fractions were pooled, concentrated, and applied to a Mono-S column (Pharmacia) in a high-performance liquid chromatography system equilibrated with 50 mmol/L sodium acetate (pH 4.8) and 0.05% (wt/vol) Tween 80. A linear gradient (0% to 100%) was formed with a buffer containing 50 mmol/L sodium acetate, 0.05% (wt/vol) Tween 80, and 500 mmol/L NaCl. The IL-2 eluted at 270 mmol/L NaCl as a sharp peak. The specific activity of this peak was 2.5×10^6 units/A280 nm unit.

IL-2 Assay

IL-2 was measured in a microassay according to Gillis et al.¹⁷ Cells (5×10^3 cells/well) of a cloned IL-2-dependent murine cell line (CTLL) were cultured in IMDM supplemented with 5% (vol/vol) FCS, penicillin (100 U/mL), streptomycin (100 μg /mL), and 2-mercaptoethanol (5×10^{-5} mol/L). After 16 hours, the cells were labeled with 0.2 μCi (≈ 7.4 kBq) ^3H -thymidine (2 Ci/mmol) and cultured for another four hours before harvesting. The IL-2 concentration was related to a standard IL-2 preparation where 1 unit of IL-2 was defined as the amount of growth factor that caused half-maximal ^3H -thymidine incorporation.

Culture Conditions and Reagents

B cells from B-PLL patient W.V. (5×10^4 cells/well) were cultured in flat-bottom microtiter plates (Nunc 96F) (0.2 mL) with IMDM supplemented with 5% vol/vol heat-inactivated human pooled serum, 5×10^{-5} mol/L 2-mercaptoethanol and antibiotics. The cells were harvested after four days after a pulse of ^3H -thymidine (0.2 μCi /well = 7.4 kBq/well, specific activity 2 Ci/mmol) was added during the last 16 hours of culture. The results are given as the mean ^3H -thymidine uptake (counts per minute) of triplicate cultures. PMA (CMC Cancer Research, Katonah, NY) was used at 1 ng/mL. Anti-T3 monoclonal antibodies (Mab) (CLB T3/4.2a) ($\gamma 2\alpha$) and anti-IL-2 receptor Mab CLB IL-2 R/1 were made in our institute. Recombinant IFN- γ (IF-RC 1001 XXG AMT 2A1A) was obtained from Boehringer (Ingelheim am Rhein, FRG) and contained 10^6 U IFN- γ /mL. Rabbit anti-IFN- γ serum was a kind gift from Dr P.H. van der Meide (TNO, Rijswijk, The Netherlands) and has been described.¹⁸

RESULTS

The Effect of Conditioned Medium on the Proliferation of B-PLL Cells and Normal B Cells

When the phorbol ester PMA was added to T cell-depleted B cells from B-PLL patient W.V., a distinct proliferative response was obtained (Table 1). This proliferation increased significantly upon addition of the supernatant (MLC-CM) from normal T cells that were stimulated with allogeneic Epstein-Barr virus (EBV)-transformed cells. In

Table 1. Induction of Proliferation in B-PLL and Normal B Cells by PMA and/or MLC-CM

Additions	Normal B Cells					
	B-PLL Cells		Donor 1		Donor 2	
	—	PMA	—	PMA	—	PMA
—	530	5,380	180	6,260	80	7,660
MLC-CM (1:15)	440	16,150	440	7,480	160	6,640
MLC-CM (1:5)	700	17,910	490	9,000	200	7,980

B-PLL cells or purified normal B cells (5×10^4 cells/well) were cultured with or without PMA (1 ng/mL) and MLC-CM. Results are given as counts per minute after a four-day culture period.

the absence of PMA, no response was obtained, and the supernatant of unstimulated T cells or EBV-transformed cells had no effect on PMA-induced B-PLL proliferation. When purified tonsil B cells from two normal individuals were cultured under the same conditions, PMA induced a proliferation comparable to the response obtained with B-PLL cells (Table 1). However, in contrast to the B-PLL cells, MLC-CM did not further enhance the outgrowth of these nonmalignant B cells. These data suggest that mitogen-free MLC-CM contains putative factor(s) that specifically act on the proliferation of PMA-stimulated cells from patient W.V. but not on nonmalignant B cells.

MLC-CM-Induced Enhancement of B-PLL Cell Proliferation Is Independent of IL-2

Previously, IL-2 has been shown to stimulate the proliferation of preactivated normal^{4,5} and malignant B cells.^{7,9} Because MLC-CM contained a small amount of IL-2 (4 U/mL), we investigated whether IL-2 was involved in the proliferative response of the B-PLL cells. Table 2 shows that the addition of a serial dilution of MLC-CM to PMA-stimulated neoplastic B cells resulted in a dose-dependent increase in proliferation whereas a dose of purified IL-2 that by far exceeded the amount present in MLC-CM had no effect. Upon the addition of Mabs against the IL-2 receptor, the MLC-CM-induced proliferation was not affected whereas the response of T cells from healthy individuals was completely blocked. In contrast to PMA-stimulated T cells, no IL-2 receptors were expressed on the B-PLL cell membrane before or after PMA/IL-2 stimulation (not shown). The combined data indicate that IL-2, either alone or as a

Table 2. Role of IL-2 in B-PLL Proliferation to PMA and MLC-CM

Cell Source	Added Stimulus	CLB IL-2 R/1	
		—	+
B-PLL cells	—	3,820	4,800
	MLC-CM, 1:120	8,920	11,400
	1:60	14,430	13,390
	1:30	20,590	19,870
T cells	IL-2, 10 U/mL	4,550	3,200
	—	5,820	1,150
	IL-2, 10 U/mL	11,900	780

B-PLL cells (5×10^4 /well) and normal T cells (3×10^4 /well) were cultured with PMA (1 ng/mL). To these cultures, either MLC-CM or IL-2 was added in the presence or absence of CLB IL-2 R/1 Mab (ascites, 1:2,000). Results show counts per minute after four days of culture. In the absence of PMA always less than 750 cpm were obtained.

cofactor, is not the active principle in MLC-CM responsible for the observed stimulatory effect on these B-PLL cells.

Analysis of MLC-CM by Gel Filtration

After concentration of MLC-CM, the molecular characteristics of the growth factor for these B-PLL cells were analyzed by gel filtration. Figure 1 shows that the growth factor activity eluted in two distinct peaks, which coincided with a molecular mass of approximately 40 and 20 kilodaltons (kD). When the different fractions were tested for the presence of IL-2, one peak of activity eluted together with the second peak of B-PLL growth factor activity at 15 to 20 kD. This finding is in agreement with the previously described molecular weight (MW) for IL-2.² Thus, it appears that the cofactor is not identical to IL-2 and acts independently from this lymphokine.

IFN- γ Induces B-PLL Proliferation in the Presence of PMA and Is the Active Principle in MLC-CM

In a search for a well-defined lymphokine that could be responsible for the observed B-PLL proliferation, we focused on IFN- γ . The molecular mass of IFN- γ is 20 and 25 kD for the two different glycosylated states of the monomeric form and 40 to 50 kD for the dimeric configuration.¹⁹ Considering the data in the previous section, we tested whether IFN- γ obtained by recombinant DNA technology (recIFN- γ) had any activity in this assay system. Figure 2 clearly demonstrates the potent stimulatory effect of this material, which was active only in the presence of PMA. To test whether indeed IFN- γ was the active principle in MLC-CM, we

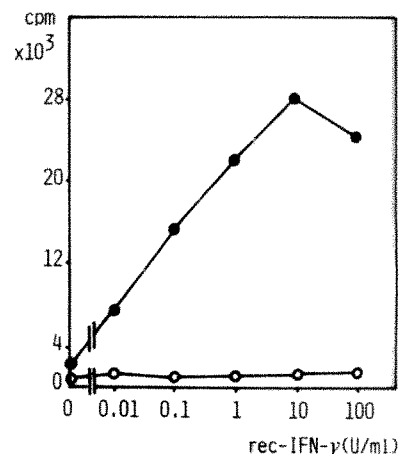


Fig 2. B-PLL proliferation induced by recIFN- γ . B-PLL cells (5×10^4 /well) were cultured with (●—●) or without (○—○) PMA (1 ng/ml) and a serial dilution of recIFN- γ . Results show ^{3} H-thymidine incorporation at day 4.

studied the effect of anti-IFN- γ antibodies on the MLC-CM-induced enhancement of B-PLL cell proliferation (Fig 3). Both IFN- γ and MLC-CM responses were blocked by these antibodies, whereas the proliferation of the neoplastic B cells to PMA alone was not affected. Incubation of MLC-CM for 30 minutes at 56°C, which is known to inactivate IFN- γ ²⁰ completely abrogated its effect (data not shown). The combined data strongly suggest that IFN- γ in the MLC-CM preparation is responsible for the increase in proliferation of the PMA-activated B-PLL cells.

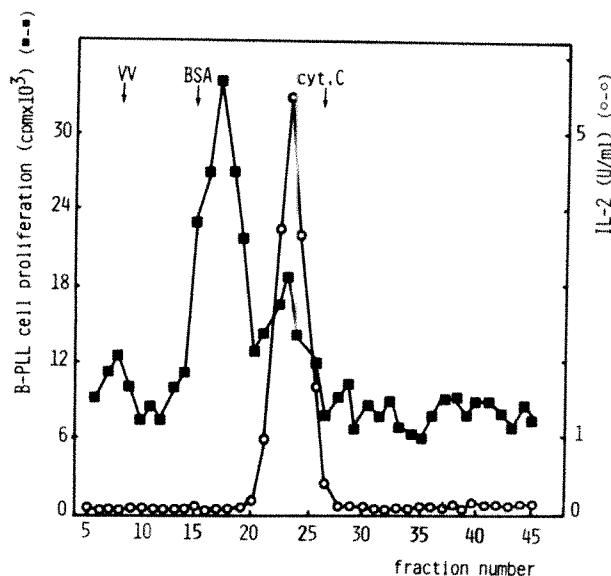


Fig 1. Analysis of MLC-CM by gel filtration. Concentrated MLC-CM was fractionated over an AcA54 column. Fractions (1:20 diluted) were tested for the capacity to induce proliferation in B-PLL cells (5×10^4 /well) in the presence of PMA (1 ng/mL). B-PLL proliferation is shown after four days of culture (■—■). PMA alone induced 7,220 cpm. In the absence of PMA, maximally 1,000 cpm were obtained. Fractions were also tested for the presence of IL-2 (○—○). VV, void volume; BSA, bovine serum albumin; cyt. C, cytochrome C.

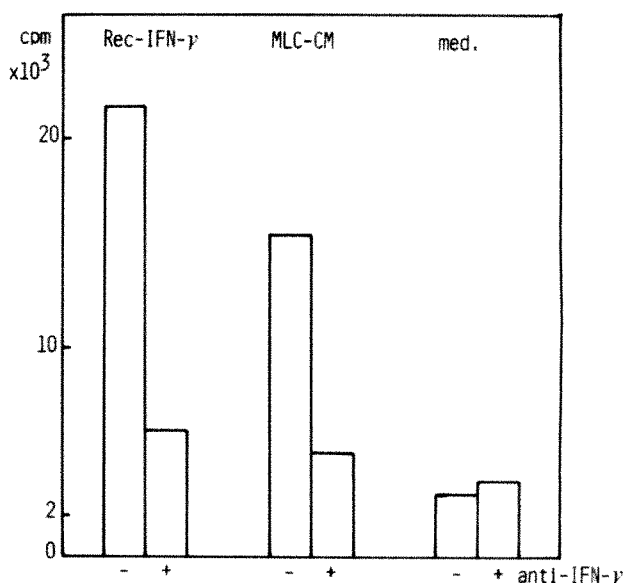


Fig 3. Effect of anti-IFN- γ antibodies on B-PLL proliferation. B-PLL cells (5×10^4 /well) in the presence of PMA (1 ng/well) were cultured with either recIFN- γ (100 U/mL), MLC-CM concentrate (1:500), or without further additions (med). These cultures were set up in the absence (—) or presence (+) of anti-IFN- γ antibodies (serum dilution, 1:10⁴). The results are given as counts per minute after 4 days of culture.

IL-2 Is Not a Growth Factor for B-PLL Cells

An indication that IL-2 did not induce proliferation in PMA-stimulated B-PLL cells was obtained by data shown in Table 2. Because the chosen PMA and IL-2 concentrations might have been insufficient, various doses of IL-2 (up to 250 U/mL) were tested in the presence of a titration of PMA. Figure 4 shows that in the presence of PMA, 250 U/mL IL-2 induced a minor increase of B-PLL cell outgrowth compared with that induced by PMA alone. This response was similar to the one with 10 U IL-2/mL (data not shown), which suggests that IL-2 concentrations above 250 U/mL would not lead to a further increase in B-PLL cell proliferation. In contrast IFN- γ at 100 U/mL showed strong B cell growth factor activity. Thus, these B-PLL cells are highly receptive to the growth-promoting activity of IFN- γ whereas IL-2 has virtually no effect.

The Effects of Preincubation With Either PMA or IFN- γ on B-PLL Proliferation

Since both PMA and IFN- γ are able to modulate membrane marker expression in normal and malignant lymphocytes,^{13,20-22} we wondered whether preincubation with either PMA or IFN- γ would induce receptors or at least susceptibility to the other stimulus. Table 3 shows that B-PLL cells preincubated with PMA for 24 hours did not respond with proliferation when recultured with IFN- γ alone. In contrast, exposure of these neoplastic B cells to IFN- γ (100 U/mL) induced responsiveness to PMA without additional IFN- γ .

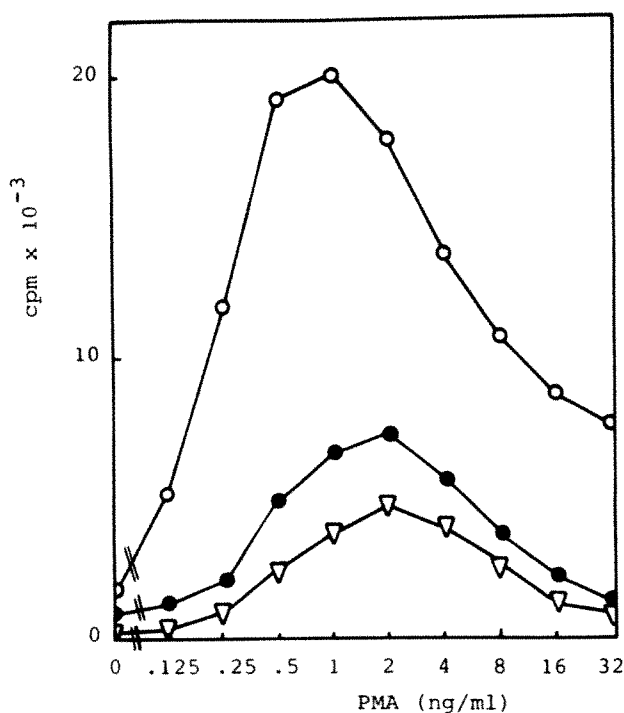


Fig 4. Effect of PMA concentration on B-PLL proliferation induced by IL-2 and IFN- γ . B-PLL cells (5×10^4 /well) were cultured with either 250 U/mL IL-2 (●—●), 100 U/mL IFN- γ (○—○), or without further additions (▽—▽). The results are given as counts per minute after four days of culture.

Table 3. Effect of Preincubation with PMA or IFN- γ on B-PLL Proliferation

24-h Preincubation	Reagent Added After Incubation	B-PLL Proliferation (cpm)	
		Experiment 1	Experiment 2
PMA	—	60	200
	PMA	2,040	6,500
	IFN- γ	80	120
	PMA + IFN- γ	10,880	22,700
	—	20	110
	PMA	1,140	3,070
IFN- γ	IFN- γ	30	200
	PMA + IFN- γ	5,370	12,500
	—	20	nt
	PMA	10,380	nt
	IFN- γ	50	nt
	PMA + IFN- γ	16,510	nt

Abbreviation: nt, not tested.

B-PLL cells (10^6 cells/mL) were incubated with either medium alone, PMA (1 ng/mL) or IFN- γ (100 U/mL) in 1-mL wells. After 24 hours, the cells were harvested, washed (three times), and recultured at 5×10^4 B-PLL cells/well in 0.2 mL with either no stimulus, PMA (1 ng/mL), recIFN- γ (100 U/mL), or both stimuli. The results are given as counts per minute after four days of reculture.

Similar results were obtained after preincubation with 1 U IFN- γ /mL (data not shown). These data suggest that either IFN- γ strongly binds to the B cell membrane and that both IFN- γ and PMA need to be present in the culture well or that IFN- γ induces modulation of these B cells, which subsequently results in an increased capacity of PMA to induce proliferation.

DISCUSSION

In the presence of PMA, several well-defined lymphokines have been shown to regulate proliferation of malignant B cells and lymphoblastoid cell lines *in vitro*.⁶⁻⁹ In the present study, we have analyzed the factor in T cell-derived conditioned medium that is responsible for the induction of proliferation in PMA-activated neoplastic B cells from a patient with B-PLL. Several lines of evidence indicate that IFN- γ is responsible for the proliferative response: (a) Gel filtration of MLC-CM resulted in two distinct peaks of activity that eluted in the 40- to 50-kD and 20-kD fractions. These MWs are reported for IFN- γ in its dimeric and monomeric form, respectively. (b) Anti-IFN- γ serum blocked the B-PLL proliferation to MLC-CM and PMA but not to PMA alone. (c) In the presence of PMA, recIFN- γ was a potent inducer of B-PLL proliferation. (d) Treatment of MLC-CM at 56°C for 30 minutes, which is known to inactivate IFN- γ ,¹⁹ abrogated the functional activity of MLC-CM in this system (unpublished results). Neither MLC-CM nor IFN- γ affected the outgrowth of PMA-stimulated or -unstimulated nonmalignant B cells, and this observation is in agreement with previous findings.¹⁰ Recently, anti- μ -stimulated nonmalignant B cells were found to proliferate in response to IFN- γ .^{11,12} In malignant B cells, IFN- γ has been reported to be a growth factor for unstimulated B-CLL cells.¹³

IL-2 either alone^{4,9,11} or as an obligatory cofactor²³ has

extensively been shown to stimulate the outgrowth of non-malignant and malignant B cells. A small amount of IL-2 was present in MLC-CM, but with the use of anti-IL-2 receptor Mabs and purified IL-2, we showed that IL-2 with or without additional PMA did not influence the proliferation of these B-PLL cells and that IL-2 was not involved in the proliferative capacity of MLC-CM. Additional IL-2 did not increase the stimulatory effect of IFN- γ on the B-PLL cell proliferation. Similar results were recently obtained by Romagnani et al in nonmalignant B cells.¹¹

Apart from B-PLL W.V. cells used in this study, we tested the functional capacity of cells from four other B-PLL patients to respond to T cell-derived signals.^{14a} Although PMA induced proliferation in all B-PLL, only cells from patient W.V. showed an increased response in the presence of MLC-CM. Three of five B-PLL were able to actively secrete immunoglobulins in vitro, thus illustrating the functional heterogeneity of this type of leukemia, just as previously described for B-PLL.^{8,9} This series is of course too small to draw any definite conclusions about the unique proliferative capacity of B-PLL W.V. cells. The relatively high expression of activation marker 4F2 on the cells of patient W.V. (Sauerwein et al, submitted) might be a clue but needs further evaluation.

In the absence of PMA, neither IFN- γ nor MLC-CM were able to induce B-PLL proliferation. PMA has been shown to change the phenotypic marker expression of malignant B cells^{21,22} and to induce putative B cell growth factor receptors.¹⁰ In the same way, PMA could have induced

susceptibility to IFN- γ in our system. However, when B-PLL cells were preincubated with PMA (up to a period of 72 hours), subsequently washed and exposed to IFN- γ , no proliferative response was found. In contrast, preincubation with IFN- γ resulted in responsiveness to PMA. IFN- γ is known to induce changes in the market profile of the cell membrane such as an increase in HLA class II expression.¹⁹ Although incubation with either PMA or IFN- γ alone induced no particular changes on the membrane of these B-PLL cells, the combination resulted in increased cell volume and HLA class II and 4F2 expression (unpublished results). This suggests that only the stimulating combination of PMA and IFN- γ can push the cells to a higher activation state. IFN- γ might have such a high affinity for these neoplastic B cells that it cannot be simply washed away from the cell membrane after preincubation.

In conclusion, by analyzing the growth-promoting activity of T cell-derived supernatants for neoplastic B cells, we found that IFN- γ was a growth factor for these B-PLL cells in the presence of PMA. This observation should be taken into consideration when IFN- γ is considered as a therapeutic tool for patients with such neoplasias.

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Analysis of CD1 Molecules on Thymus Cells and Leukemic T Lymphoblasts Identifies Discrete Phenotypes and Reveals That CD1 Intermolecular Complexes Are Observed Only on Normal Cells

By Martine Amiot, Helene Dastot, Michel Schmid, Alain Bernard, and Laurence Boumsell

We looked at the surface expression of the three distinct human thymic cell surface differentiation antigens, CD1a, CD1b, and CD1c, that presently define the first cluster of differentiation (CD) on the cells from 34 patients with acute T cell malignancies. We also studied the expression of other T cell-restricted molecules, including the T cell receptors, on these cells. Our results confirm the extensive phenotypic heterogeneity of the cells from acute T cell malignancies, which contrast with the more limited phenotypic diversity of subacute or chronic T cell malignancies. Our study of normal children and fetal thymus cells shows that the extensive phenotypic heterogeneity of the malig-

nant cells reflects the heterogeneity of the thymic subpopulations and shows that most of the phenotypes observed on malignant T cells have a normal counterpart, particularly in the fetal thymus. Moreover, we demonstrate that the CD1a molecules, which can form three different types of noncovalent intermolecular complexes on the surface of normal thymus cells, do not form any noncovalent intermolecular complexes on the surface of leukemic cells. We also show that CD1a molecules can form covalent intermolecular complexes with CD8 molecules on some but not all malignant cells.

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THE FIRST cluster of differentiation¹ (CD1) defines at least three distinct human thymic cell surface differentiation antigens, CD1a (49 kilodaltons [kD]),² CD1b (45 kD)^{3,4} and CD1c (43 kD).⁵ Their expression, similar to the murine class I gene product Ta, is restricted to cortical thymocytes, to cells from some leukemic patients,^{6,7} and to dendritic cells in the skin.^{8,9} The CD1 molecules display a heavy chain of approximately 45 kD noncovalently attached to β_2 -microglobulin (β_2 M). The three CD1 heavy chains differ in isoelectric points,^{4,10,11} differ slightly in their molecular weight,^{4,10,11} and differ immunologically.^{4,12,13} We have previously shown, by 2D-peptide maps that the CD1a 49-kD and the CD1b 45-kD heavy chains are more homologous to each other ($\approx 40\%$) than they are to the CD1c 43-kD heavy chain ($\approx 12\%$ homology) and that only one tyrosine containing peptide is common to the three heavy chains.^{14,15}

The association of the three heavy chains with β_2 M differs: the CD1c heavy chain is strongly associated with β_2 M,^{4,16,17} but the CD1b and CD1a heavy chains are weakly associated with β_2 M.^{4,16-18} Moreover, it was shown that some of the CD1a heavy chains devoid of β_2 M^{19,20} are in covalent association with some of the CD8 molecules. We have demonstrated that on the surface of normal thymus cells the CD1a molecules can form four different types of intermolecular complexes by associating noncovalently either with CD1c

molecules, with CD1b molecules, with HLA class I molecules, or by covalently associating with CD8 molecules.^{14,15} In contrast we showed that, on the cell surface of a T leukemic line in culture, CD1a and CD1c molecules are not associated.

It has recently been reported that the CD1 molecules are encoded by a new family of major histocompatibility complex (MHC)-related genes^{21,22} encoded on human chromosome 1. It is of note that these genes have the same level of homology with all members of the MHC gene family, and thus they do not show higher homology with thymus leukemia (TL) genes. Yet the CD1 molecules share other characteristics such as tissue distribution with the TL gene products Ta. Ta is a rare example of a cell surface glycoprotein for which modifications on leukemic cells have been well documented both at the level of the regulation of its expression²³ and its structure.²⁴

We report here the results of our investigation of a large panel of leukemic T cells that were freshly isolated from patients. We have also investigated fetal and child thymus cells to address the following questions: Are the three CD1 molecules always coexpressed? Are the CD1 molecules in association with other cell surface molecules delineating phenotypes that are characteristics of malignant T cells? Do the CD1a molecules on fresh malignant T cells form any covalent or noncovalent intermolecular complex?

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies (MoAbs) CD1a "L119," "L404," and "L544," "CD1c" "L161," and CD8 "L533" were obtained in our laboratory from the fusion of cells from the NS1 myeloma cell line to spleen cells from Biozzi high-responder mice.²⁵ The CD1b MoAbs "4A7.6," "7C4," "WM25," and "NU-T2" were kindly provided through the exchanges of the Third International Workshop on Human Leucocyte Differentiation Antigens by Drs C. Mawas, W. Knapp, K. Bradstock, and K. Sagawa respectively. MoAbs "L119," "L404," and "L544" have been shown to recognize three different epitopes of the CD1a molecule; MoAb CD1c "L161" recognizes the same epitopic group of CD1c molecules as CD1c "M241"; MoAbs "4A7.6," "WM25" or "7C4," and "NU-T2" recognize two different epitopes of the CD1b molecule; and MoAb "L533" recognizes CD8.¹⁵ MoAbs CD3 "OKT3," CD4 "Leu 3a," W6/32 which recognize a monomorphic determinant of the HLA class I molecules, and WT31, which recognizes a monomorphic

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determinant of the α - β T cell receptor heterodimer, have been extensively described elsewhere.^{7,26-28}

Cells and cell lines. Thymus fragments were obtained from children undergoing cardiac surgery or from fetuses at the time of postmortem examination. Gestational ages as determined by crown-rump length or menstrual record ranged from 10 to 22 weeks. After isolation of the thymic cells, the cells were cryopreserved as previously described.²⁵ For immunofluorescence assays the thymus cells were used fresh or after cryopreservation with identical results.

The human leukemic T cell lines were continuously grown in tissue culture media supplemented with 10% normal human AB sera to avoid exchanges with xenogeneous β_2 M as described.^{16,17}

Children and adults were investigated in the present study. They were cared for at the Institut Gustave Roussy at Villejuif or the Hôpital Saint-Louis in Paris. In all cases, tumor cells were collected after informed consent and before initial chemotherapy. The diagnosis of lymphoblastic lymphoma (LL) was established by several strict criteria. All patients presented with tumor masses, and tumor cells from lymph nodes and mediastinal masses or pleural effusions were characterized by using the standard morphological and histochemical criteria. All patients underwent bone marrow aspiration from at least four different and distant sites. Whenever a patient had more than 25% lymphoblasts in one of the bone marrow smears, he was considered ambiguous for categorization in either leukemic or lymphomatous diseases and therefore was systematically removed from the study. ALL patients retained in the study had more than 95% bone marrow involvement and in most cases had blood involvement. Cells from pleural effusion, lymph nodes, bone marrow, or blood were isolated by Ficoll-Hypaque density gradients and centrifugation. Tumor cells were readily distinguishable from normal lymphocytes by Wright-Giemsa morphology, and all neoplastic preparations analyzed had more than 90% abnormal cells. Isolated tumor cells were stored in liquid nitrogen according to a previously described method.²⁵

Immunofluorescence assays. Indirect immunofluorescence assays were performed by using a fluorescein isothiocyanate (FITC)-antimouse Ig from Meloy Laboratories (Springfield, VA). Fluorescence was read by using a Facstar microfluorometer (Becton Dickinson, Mountain View, CA). For two-color immunofluorescence experiments, thymocytes (1 to 1.5×10^6) were incubated with the first MoAb for 30 minutes at room temperature. After two washes, an appropriate dilution of an FITC-conjugated Fab fragment of a goat anti-mouse Ig was added to the cell pellet and incubated for 30 minutes. The cells were washed again before an optimal amount of a biotin-conjugated MoAb was added and incubated for 30 minutes. The cells were washed again before an appropriate amount of phycoerythrin (PE)-conjugated streptavidin was added and incubated as recommended by the manufacturer (Becton Dickinson). After final washes, the cells were resuspended in 0.3 mL of cold 1% formalin solution in phosphate-buffered saline. Samples were stored at 4°C in the dark until analysis. Controls consisted of thymocytes stained with isotype-matched control MoAbs that did not specifically react with human leukocytes. On several occasions an FITC-conjugated MoAb was used instead of the first MoAb plus an FITC-conjugated Fab fragment of a goat antimouse Ig with identical results. Fluorescence emissions of FITC and PE were distinguished by using 530 ± 15 and 575 ± 12.5 bandpass filters. The overlap in FITC and PE fluorescence emissions was corrected by using an electronic compensation network.

Labeling and immunoprecipitations of cell surface proteins. Cells were surface labeled with ^{125} I (2 mCi/20 to 50×10^6 cells) with lactoperoxidase and four additions of H_2O_2 during a 20-minute incubation. The cells were then lysed in standard lysis buffer (50 mmol/L Tris-HCl, pH 8.0) containing 1% (wt/vol) Nonidet P40 (NP40), 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5

mmol/L KCl, and the following protease inhibitors: 1 mmol/L phenylmethylsulfonyl fluoride and 0.2 mol/L iodoacetamide (Sigma Chemical Co, St. Louis). Cells were solubilized at a concentration of 25×10^6 cells/mL of lysis buffer for 45 minutes on ice. The lysates were centrifuged at 12,000 g, and the supernatants were stored at -70°C until needed. The cell lysates were then precleared by using an irrelevant ascites, a goat antimouse Ig, and the *Staphylococcus* Cowan 1 strain. Immunoprecipitations were performed by using MoAbs cross-linked to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described.²⁹ The precipitates were extensively washed before elution in sodium dodecyl sulfate (SDS) sample buffer and electrophoresis.³⁰

Electrophoresis and autoradiography. SDS 12% polyacrylamide gel electrophoresis (PAGE) was carried out on 30-cm-long vertical slab gels according to the modification of the Laemmli procedure³⁰ and were run under reducing conditions.

For autoradiography of ^{125} I-labeled material, Kodak XAR x-ray film was used in combination with intensifier screens (Cronex Lightning Plus; Dupont Chemical Co, Newton, CT) and the dried gels exposed at -70°C .

2D-peptide map analysis of separated CD1 heavy chains. After 1D SDS electrophoresis, bands corresponding to the heavy chains were separately cut from dried gels, rehydrated in 50 mmol/L NH_4HCO_3 containing 0.05% SDS, incubated for 15 hours at 37°C , and digested with pepsin as described.^{31,32} Peptide aliquots (10×10^3 cpm in 2 to 4 μL) were spotted on 20×20 -cm silica thin-layer chromatography (TLC) plates. Electrophoresis was performed in 5% acetic acid and 15% formic acid, and chromatography was carried out in 40% *n*-butanol, 8% acetic acid, and 28% pyridine and was followed by autoradiography.

RESULTS

Reactivity of CD1 MoAbs with cells from patients with acute T cell leukemias and T cell LL. The specificities of the antibodies used for this study have been extensively described elsewhere. In brief, MoAb L404 recognizes the CD1a molecule, and MoAb WM25 recognizes the CD1a molecule, whereas MoAb L161 recognizes the CD1c molecule.¹³ We analyzed the reactivity of these MoAbs on cells from 34 patients with known T cell malignancies. Among these patients, 14 had LL and 20 patients had acute lymphoblastic leukemia (ALL). Nineteen of these patients were selected for this study because their cells, in addition to being stained by CD5, CD7, and/or CD2 MoAb, were stained by CD1a MoAb. The other 15 patients were selected at random among a large panel of T cell ALL or T cell LL, which had been shown not to be stained by CD1a MoAb. Immunophenotypic data are presented in Table 1. Among the 19 patients with CD1a-positive cells, all but one (patient 81-35) had cells also expressing CD1b and CD1c molecules. Among the 15 patients with CD1a-negative cells, three patients had cells expressing CD1b molecules only (81-125, 81-28, LS 477) and one patient had cells coexpressing CD1b and CD1c molecules (83). Results also showed that, among the cells coexpressing the three CD1 molecules, the relative density in the three CD1 molecules varied, ranging from a phenotype with the highest expression in CD1a to a phenotype with the highest expression in CD1b (results not shown).

It can also be seen in Table 1 that the expression and the relative densities (not shown) of any one of the CD1 molecules did not correlate with the expression of the T cell

Table 1. Immunophenotypic Data for Leukemic Cells

Case No.	Cluster or Antibody Designation						
	CD1a	CD1b	CD1c	CD3	WT31	CD4	CD8
LS 366	+	+	+	—	NT	—	+
LS 544	+	+	+	—	—	+	+
52	+	+	+	—	NT	+	+
83-45	+	+	+	—	NT	+	+
81-108	+	+	+	—	NT	+	+
LS 199	+	+	+	—	NT	+	+
83-66	+	+	+	—	NT	+	+
84-77	+	+	+	—	NT	+	+
LS 92	+	+	+	—	NT	+	—
82-57	+	+	+	—	NT	+	+
81-35	+	+	—	—	—	—	+
81-125	—	+	—	—	NT	—	+
83	—	+	+	—	NT	+	+
81-28	—	+	—	—	NT	+	+
LS 477	—	+	—	—	NT	+	+
LS 29	+	+	+	+	+	+	+
LS 76	+	+	+	+	+	+	+
LS 86	+	+	+	+	+	+	+
LS 55	+	+	+	+	—	+	+
LS 114	+	+	+	+	+	+	+
82-82	+	+	+	+	+	+	+
408	+	+	+	+	—	+	+
LS 113	+	+	+	+	+	+	+
LS 507	—	—	—	+	NT	+	+
82-37	—	—	—	+	NT	NT	+
81-56R	—	—	—	+	—	—	+
81-132	—	—	—	+	—	—	—
82-14	—	—	—	—	NT	—	—
81-116	—	—	—	—	NT	NT	—
81-36	—	—	—	—	NT	—	—
45	—	—	—	—	NT	—	—
62	—	—	—	—	NT	—	+
LS 134	—	—	—	—	NT	NT	NT
LS 433	—	—	—	—	—	—	—
HD-Mar	+	+	+	+	NT	—	—
MOLT 4	+	+	+	—	NT	—	+
JM	+	+	+	+	NT	+	+
HPB-ALL	—	+	—	+	NT	+	+
KE 37	—	—	—	—	NT	+	+

Abbreviations: +, $\geq 25\%$ of the cells were stained by the MoAb; —, $< 25\%$ of the cells were stained by the MoAb; NT, not tested.

receptor and associated cell surface detected by WT31 or CD3 MoAbs. WT31 MoAb has previously been shown to recognize a monomorphic determinant of the T cell receptor α - β heterodimer, whereas CD3 recognizes a molecular complex associated with the T cell receptors encoded by the α - β or the γ genes.²⁸ The cells from only six of ten patients that were stained by CD3 MoAb were also stained by WT31, whereas the cells from four patients (LS 55, 408, 81-56R, 81-132) were not stained by WT31. Among these four patients only two (LS 55, 408) had cells also stained by CD1 MoAb. It is of note that the cells from these two patients were also stained both by CD4 and CD8 MoAbs. In contrast the cells from the other two patients (81-56R, 81-132), which were CD3-positive, WT31-negative, and CD1-negative, were stained by CD8 MoAb only and not by CD4 or CD8 MoAbs. It is also of note that among the 15 patients

with CD1b-positive, CD3-negative cells, four patients had cells expressing CD4 or CD8 molecules only. This is in contrast with the fact that the cells in eight of eight patients with CD1-positive, CD3-positive cells were coexpressing CD4 and CD8 molecules.

Reactivity of CD1 MoAbs with T cell lines. Studies of the reactivities of the same MoAbs on five selected T cell lines also revealed additional patterns of staining as shown in Table 1. KE 37 cells were stained neither by CD1 nor by CD3 MoAbs; HPB-ALL cells were stained brightly only by CD3 and CD1b MoAbs; JM cells were stained brightly by CD3 MoAb, dimly by CD1c and CD1b MoAbs, and hardly stained by CD1a MoAb; finally, both on HD-Mar cells, which were dimly stained by CD3 MoAb, and MOLT4 cells, which were not stained by CD3 MoAb, the relative density of CD1 molecules was highest for CD1c, intermediate for CD1a, and lowest for CD1b. These results were obtained by using different MoAbs recognizing the same or different epitopes of the three CD1 molecules.

Taken together these results indicate that malignant T cells show a high degree of heterogeneity, which contrasts with the more limited phenotypic diversity of subacute or chronic T cell malignancies.³³ Characteristics of malignant cells, often initially considered to reflect aberrant differentiation, have in most instances been shown later to reflect clonal selection of cell types that are normally infrequent or transitory. Therefore we investigated the subpopulations of cells delineated in fetal and child thymus with the same battery of MoAbs.

Subpopulations of cells delineated within normal child thymus by using CD1 and CD3 MoAbs. By simple labeling experiments we observed that CD1a was the most abundant molecule, CD1b had an intermediate density, and the CD1c molecule was the least abundant on normal human thymus cells. We next observed, by two-color fluorescence experiments revealed by flow microfluorimetry, that the cells having the highest relative density in CD1a molecules also had the highest relative density in CD1b and CD1c molecules as shown in Fig. 1. We also observed by two-color fluorescence experiments that in children most normal cortical thymus cells, as shown in Fig. 1, coexpressed the three CD1 molecules. However, minor populations (2% to 5%) of cells expressing only one or the other CD1 molecule were also observed, and finally, a subpopulation of cells (10% to 15%) expressed none of the CD1 molecules. CD1 molecules have been shown to correspond to less mature cortical thymus cells, whereas CD3 molecules are known to be associated on the cell surface with T cell receptors' heavy chains. We thus investigated in more detail the subpopulations of cells delineated by these markers. Two-color fluorescence experiments, shown in Fig 2, with CD3 and CD1a MoAbs revealed two major and several minor subpopulations of thymus cells. Similar results were obtained after double-labeling experiments with CD3 and CD1b or CD3 and CD1c MoAbs. We also obtained similar results when WT31 MoAb was used instead of CD3 MoAb (results not shown). From the results of systematic two-color fluorescence performed with CD1a, CD1b, CD1c, and CD3 MoAbs, six subpopulations of cells were delineated in child thymus as shown in Fig 3. The two

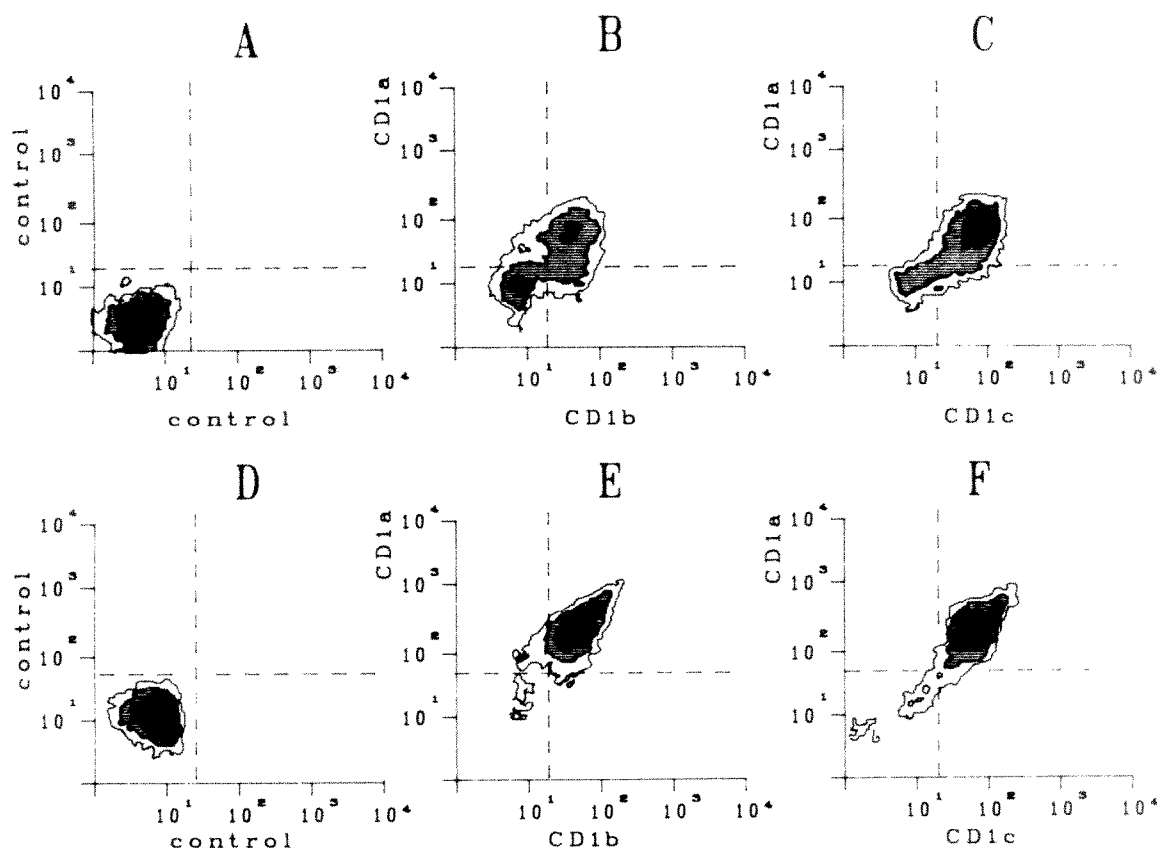


Fig 1. Correlated expression of CD1a and other CD1 molecules on human thymocytes. Thymus cells from a 16-week-old fetus (panels A, B, and C) or from a 2-month-old child (panels D, E, and F) were stained with (panels A and D) control MoAbs that do not recognize human thymus cells, an FITC-conjugated Fab fragment of a goat antimouse Ig, a biotin-conjugated MoAb with irrelevant specificity, and streptavidin-PE; (panels B and E) CD1b 4A7.6 MoAb, an FITC-conjugated Fab fragment of a goat antimouse Ig, a biotin-conjugated CD1a L404 MoAb and streptavidin-PE; (panels C and F) CD1c L161 MoAb, an FITC-conjugated Fab fragment of a goat antimouse Ig, a biotin-conjugated CD1a L404 MoAb, and streptavidin-PE. Samples were analyzed by flow cytometry, and data are displayed as two-dimensional contour plots. On the basis of control samples (panels A and D), contour plots were divided into quadrants identifying unstained cells (lower left quadrants), cells stained with both MoAbs (upper right quadrants), cells stained with CD1a MoAb only (upper left quadrants), or with the other CD1 MoAb only (lower right quadrants). In the control samples, <2% of the cells were stained.

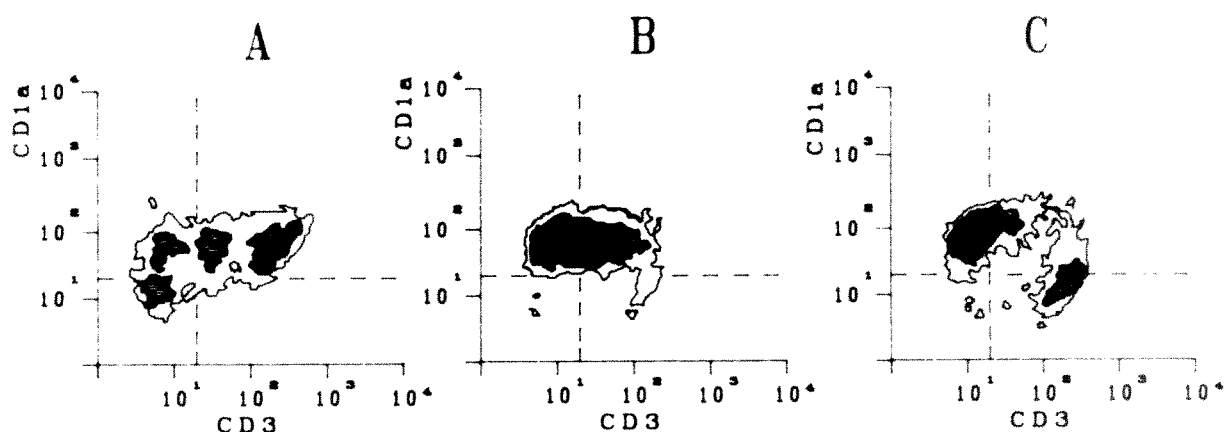


Fig 2. Correlated expression of CD1a and CD3 molecules on human thymocytes. Thymus cells from a 16-week-old fetus (panel A), from a 22-week-old fetus (panel B), or from a 2-month-old child (panel C) were stained with control MoAbs that do not recognize human thymus cells, an FITC-conjugated Fab fragment of a goat antimouse Ig, a biotin-conjugated MoAb with irrelevant specificity, and streptavidin-PE (panels A and D from Fig 1 are not shown); CD3 OKT3 MoAb, an FITC-conjugated Fab fragment of a goat antimouse Ig, a biotin-conjugated CD1a L404 MoAb, and streptavidin-PE (panels A, B, and C). Samples were analyzed by flow cytometry, and data are displayed as two-dimensional contour plots. On the basis of control samples (panels A and D), contour plots were divided into quadrants identifying unstained cells (lower left quadrants), cells stained with both MoAbs (upper right quadrants), cells stained with CD1a MoAb only (upper left quadrants), or with CD3 MoAb only (lower right quadrants). In the control samples, 2% of the cells were stained.

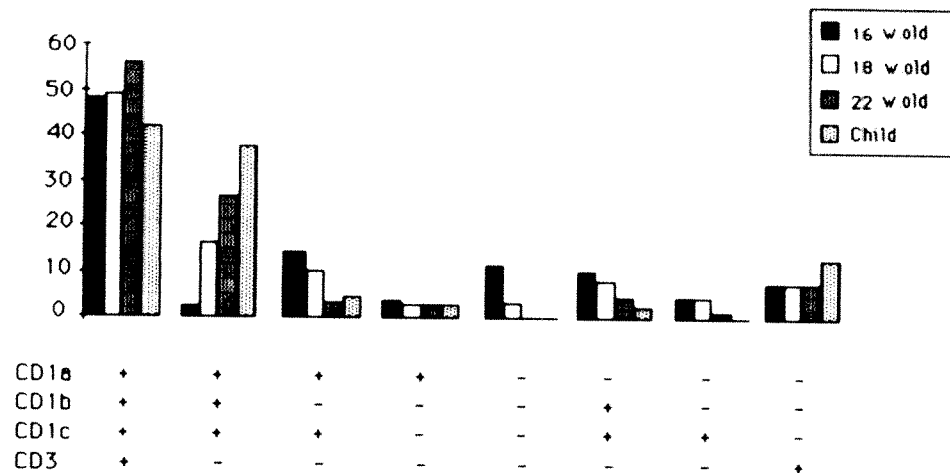


Fig 3. Subpopulations of thymus cells delineated by systematic two-color fluorescence analysis using CD1 and CD3 MoAbs. Thymus cells from a 16-week-old fetus, from an 18-week-old fetus, from a 22-week-old fetus, or from several normal children were analyzed by systematic criss-cross two-color fluorescence with CD1a, CD1b, CD1c, and CD3 MoAbs. From the overall results we could calculate the percentages of cells expressing 0, 1, 2, 3, or all of these molecules. Thus eight subpopulations of cells were defined in 16- and 18-week-old fetal thymus, whereas six subpopulations of cells were defined in child thymus. The percentage of the cells in each subpopulation is indicated by the height of the column.

major subpopulations were stained by CD1 MoAb only (40%) or by CD1 and dimly by CD3 MoAbs (40%).

Subpopulations of cells delineated within fetal thymus by using CD1 and CD3 MoAbs. We used the same MoAb to investigate the cells obtained from the thymus of 10- to 22-week-old fetuses. Table 2 shows that we observed an increase in the percentage of CD1-positive cells between 10 and 22 weeks of age. This increase concerned the three CD1 molecules. Moreover, three subpopulations were delineated in the thymus at 10 and 16 weeks of gestation by CD1a MoAb. One third of the cells were brightly stained by CD1a MoAb, another third of the cells were dimly stained by CD1a MoAb, and the last third was not stained by this MoAb. The percentage of cells stained by CD3 and CD8 MoAbs also increased with the age of the fetus. It is also of note that smaller percentages of thymic cells were labeled with WT31 than with CD3 MoAbs in young fetal thymus. We could also investigate the subpopulations of thymic cells from the 16- to 22-week-old fetuses by two-color immunofluorescence. The cell subpopulations delineated in a thymus from a 22-week-old fetus were essentially the same as in the child thymus, although their proportions varied. In particular, the proportion of the CD3+, CD1- subpopulation was smaller,

whereas the proportion of the CD3+, CD1+ subpopulation was higher than in child thymus, as can be seen from Fig. 2. Additional differences in terms of subpopulations were observed in thymus obtained from younger fetuses. Namely, larger subpopulations of cells were stained by one and not by the other CD1 MoAbs as shown in Figs 1 and 3. In addition, among the cells having the highest density in one CD1 molecule, a proportion had a low density for another CD1 molecule (Fig 1). Moreover, as can be seen in Figs 2 and 3, most of the cells that express CD3 molecules also expressed CD1a molecules, and thus the subpopulation of cells expressing only CD3 is small and increases late in ontogeny. Conversely, the population of cells expressing neither CD1 nor CD3 molecules decreases during ontogeny (Fig 3).

Lack of intermolecular complexes including CD1a molecules at the surface of leukemic cells. We have previously shown that, on the surface of normal child thymus cells, CD1a molecules can associate with either of four different molecular species, thus forming a complex that we refer to as intermolecular complexes.^{14,15} Thus on thymus cells, CD1a molecules can either be free or form intermolecular complexes with CD1b, CD1c, HLA class I, or CD8 molecules. Moreover, although CD1a is covalently associated to CD8, the three other types of intermolecular complexes involve noncovalent interactions between the two different molecules of the intermolecular complex. The molecular components of the intermolecular complexes between CD1a and CD8 or between CD1a and CD1c molecules can be dissociated on SDS-PAGE and are observed on autoradiograms of CD8 or CD1c immunoprecipitates run under reducing conditions on SDS-PAGE. As shown in Fig 4, the 49-kD heavy chain of the CD1a molecule is observed in the CD8 immunoprecipitate and in the CD1c immunoprecipitate. In contrast, the molecular components of the intermolecular complexes between CD1a and CD1b or between CD1a and HLA class I molecules can only be discriminated by 2D-peptide maps of the

Table 2. Fetal and Child Thymocytes Marked With MoAb

CD or MoAb Designation	10 wo	16 wo	18 wo	22 wo	Child
CD1a	65	68	85	85	80
CD1b	65	62	75	84	80
CD1c	69	78	78	90	80
CD3	57	65	62	65	65
WT31	43	52	NT	NT	64
CD8	72	NT	86	91	80

Results are the percentage of positive cells read by microfluorometry after staining with MoAb and FITC-goat antimouse Ig.

*Thymus cells from a 10-week-old fetus.

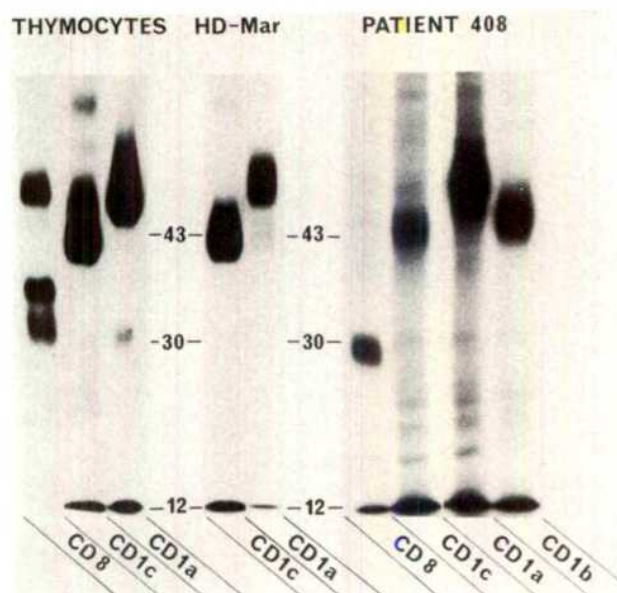


Fig 4. SDS-PAGE analysis of immunoprecipitates obtained from NP-40 lysates of ^{125} I-surface labeled cells from normal child thymus, the HD-Mar cell line, or patient 408 with T cell ALL. SDS-12% PAGE analysis of immunoprecipitates was carried out on 30-cm-long vertical slab gels and run under reducing conditions. The thymus cell lysate was immunoprecipitated by CD8 L533, by CD1c L161, or by CD1a L404 MoAbs. HD-Mar cell lysate was immunoprecipitated by CD1c L161, or CD1a L404 MoAbs. The cell lysate from patient 408 with T cell ALL was immunoprecipitated by CD8 L533, CD1c L161, CD1a L404, or CD1b 4A7.6 MoAbs. The CD1a 49-kD heavy-chain protein band is observed in all aforementioned immunoprecipitates performed on human thymus cell lysates, whereas it is observed only in the CD1a immunoprecipitates from cell lysates of HD-Mar and patient 408.

two heavy chains' protein band isolated from CD1b or W6/32 immunoprecipitates after SDS-PAGE, as shown in Figs 4 and 5. Moreover, we had shown that none of the CD1a MoAbs recognize the CD1a molecules in the intermolecular complexes with CD1b or CD1c or HLA class I molecules^{14,15} whereas they weakly recognize the CD1a molecules associated with CD8. Finally, we had shown that we cannot detect intermolecular complexes on the T cell line HD-Mar (Fig 4), which, as shown in Table 1, coexpresses CD1a molecules with high levels of CD1c molecules.

Therefore we investigated whether we could detect intermolecular complexes on leukemic cells freshly isolated from patients. We observed on the cells from one patient (no. 408) that we could extensively study that CD1a was not associated with CD1c molecules (Fig 4) or with CD8 (Fig 4) and no CD1a (49-kD) heavy-chain protein band was observed in these immunoprecipitates. Overall, the cells from six patients were tested for CD1a intermolecular complexes with CD8, and they were observed in only three cases. The cells from three patients were tested for CD1a-CD1c intermolecular complexes that were not observed even on the cells from one patient where the CD1a molecules formed CD1a-CD8 intermolecular complexes. Finally, the cells from two patients were tested by 2D-peptide maps for the presence of CD1a-CD1b intermolecular complexes, which, as shown in Fig 5

for one of the patients (83-45), were not detected. It is of note that in another patient (81-125) the CD1a molecules were all associated with CD8 molecules, so his cells had been classified as lacking CD1a molecules on their surface. Moreover, in this patient we could not detect CD1a-CD1b intermolecular complexes (results not shown).

DISCUSSION

We report our results of the immunophenotyping analysis of the cells from 20 patients with T cell ALL and from 14 patients with T cell LL. Our study with MoAbs recognizing seven different molecular entities confirms previous observations, including ours, on the diversity of the cell phenotype of these diseases.^{6,7} Moreover, this study also confirms that some phenotypes are more frequent in LL than in ALL.^{6,7}

The study of the CD1 molecules shows that the cells from more patients than previously thought may have a normal counterpart within the human child or fetal thymus. In particular we observed that the cells from four of 15 patients that were previously classified as very immature because they are CD1a-negative should now be classified as cortical since they are CD1b-positive. We cannot know from the relatively small number of patients tested whether this phenotype is more frequent in ALL or in LL, but among these four patients three had ALL, whereas only one had LL. We also observed in one patient cells expressing CD1a and CD1b but not CD1c molecules. Moreover, we observed variations in the relative surface densities of the three CD1 molecules: nine patients had cells that, similarly to most child thymic cells, expressed more CD1a than CD1b molecules and more CD1b than CD1c molecules. Six patients had cells expressing, similarly to some fetal thymus cells, more CD1b than CD1a or CD1c molecules. Finally, it is of note that some of the T cell lines tested expressed more CD1c than CD1a or CD1b molecules. The regulation of the expression of the CD1 molecules is poorly understood at present. Interferon has been shown to increase or to induce the expression of MHC class I and class II molecules. In contrast, studies involving mutants of the MOLT 4 T cell line have shown that interferon does not increase the expression of the CD1a molecules,³⁴ whereas our own unpublished results on the HD-Mar T cell line has shown that interferon does not increase the expression of any of the three CD1 molecules. The observation that some T cell malignancies, T cell lines, or normal thymic cells express only one or the other CD1 molecule argues for the independent regulation of the products of this newly identified family of MHC-related gene. These results are in agreement with the facts that Langerhans' cells of the skin express CD1a molecules only whereas a subpopulation of B cells expresses only CD1c molecules.^{9,35}

We report also our results of double-labeling experiments performed on fetal or child thymus cells with CD1a, CD1b, CD1c, and CD3 MoAbs. We show the extreme diversity of normal thymic cell subpopulations. Our results confirm and extend previous reports by us^{36,37} and more recent reports from others.³⁸ We had previously established the existence of at least 13 different cell subpopulations in normal child thymus.^{37,39} We show here that minor populations of cells

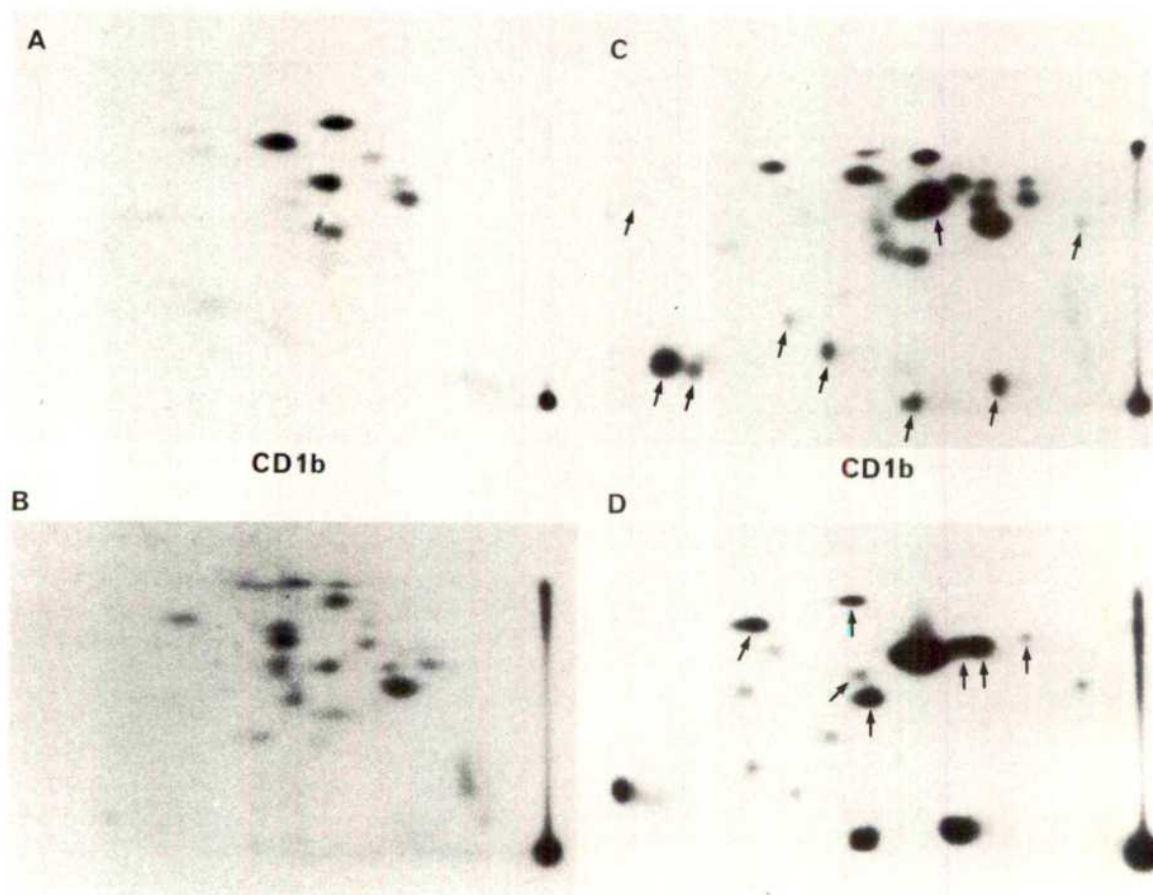


Fig 5. Comparison among ^{125}I -peptic digests of the heavy chain precipitated by CD1b 4A7.6 or CD1a L404 MoAbs from normal and leukemic cell lysates. CD1 molecules were isolated by immunoprecipitation with the relevant MoAb and separated on slab gels, as shown in Fig 4. Pepsin digests were analyzed on thin-layer silica plates by electrophoresis in the first dimension (horizontal axis) and chromatography in the second dimension (vertical axis). (A) Peptide map of the heavy chain precipitated by CD1b MoAb from the cell lysate of patient 83-45. (B) Peptide map of the heavy chain precipitated by CD1b MoAb from the cell lysate of HPB-All. (C) Peptide map of the heavy chains' protein band precipitated by CD1b MoAb from the lysate of normal thymus cells. (D) Peptide map of the heavy chains' protein band precipitated by CD1a MoAb from the lysate of normal thymus cells. Arrows in panel C indicate predominant peptides from the CD1a heavy chain that are observed in the CD1b immunoprecipitate from thymus cells (panel C) and not from leukemic cells (panels A and B). Arrows in panel D indicate peptides that are common to the CD1a and CD1b heavy chains.

expressing one or two and not the third CD1 molecules exist within child or fetal thymus and that these cell populations are larger during thymic ontogeny. We show that most cortical thymic cells in children coexpress the three CD1 molecules and that the density is the highest in CD1a molecules whereas the density is the lowest in CD1c molecules. We also show that in fetal thymus the density in CD1b or in CD1c molecules may be higher than in CD1a. Finally, we show variations both in terms of the subpopulations or the proportion of the subpopulations of cells present in thymus at 16 weeks of gestation and in children. This extends previous studies including ours with lectin probes³⁶ and others with MoAb staining on sections of early fetal thymus cells⁴⁰ that showed modification of the subpopulation of cells in the thymus during ontogeny.

Characteristics of cancer cells or leukemias have often been considered to reflect aberrant differentiation. More often they reflect clonal selection of cell types that are infrequent or transitory. This situation was particularly well

exemplified by our initial description of the expression of CD5 molecules on the surface immunoglobulin-bearing cells from patients with B cell chronic lymphocytic leukemias.^{41,42} It is now well established that these cells are normally present in subpopulations of fetal and adult B cells.⁴³ Here we show that most of the phenotypes that we observed on the malignant T cells, in terms of expression or cell surface densities of the CD1 and CD3 molecules, are normally encountered in children and/or in fetal thymus. In particular, we show here that a high proportion of patients (40%) with cells expressing, at their surface, the T cell receptor-associated CD3 molecule do not coexpress the classic $\alpha\beta$ heterodimeric structure recognized by MoAb WT31. This could indicate that their cells express the newly identified T cell receptor encoded by the γ gene.⁴⁴ Two of these four patients had cells also expressing the three CD1 molecules. It was recently reported that approximately 3% of peripheral T cells and only 0.8% of normal thymic cells are recognized by CD3 but not by WT31.⁴⁵ Moreover, a thymic cell clone with

the phenotype CD3+, WT31-, CD1- allowed the identification of the T cell receptor encoded by the γ gene.⁴⁶ Our study of early fetal thymus cells indicated that most cells expressing CD3 also expressed CD1a whereas only a proportion of the CD3+ cells were stained by WT31. This thus indicates that the normal counterpart to two of our patients' cells may be found in fetal thymus and the normal counterpart to the other two patients may be found in child thymus or in blood. We did not yet find the normal counterpart to the CD1b-positive, CD1a- and CD1c-negative cells that we observed in three patients. In keeping with the view that leukemic cells always have a normal counterpart, this may indicate that this phenotype is observed very early during thymic ontogeny or that these cells are not resident in the thymus.

We had previously shown that CD1a molecules form intermolecular complexes on the surface of normal human thymus cells by associating noncovalently with CD1b, CD1c, or HLA class I or covalently with CD8 molecules. Since we did not observe intermolecular complexes of the CD1a molecules on the surface of T cell lines in culture, we investigated whether intermolecular complexes could be detected on the surface of fresh leukemic cells. We report here that intermolecular complex formation is a rare event on fresh leukemic cells and then involves formation of a covalent CD1a-CD8 complex. Events regulating the formation of these associations between molecules are not known at present, but it can be deduced from our results that the relative density of the two components of the complex at the cell surface would not be the only regulating factor. Modification of the glycosylation of malignant cells has previously been described,⁴⁷ and it will be of interest to investigate whether the lack of association of the CD1a molecules is linked to a modified glycosylation of any of the molecules of the complex. The biologic function of the CD1a intermolecular complexes is not presently known, nor is the function of the several CD1 molecules known. It is interesting, however, that CD1a can associate with two different molecules, CD8

and HLA class I, that are thought to interact together as an associative recognition structure to the T cell receptor-nominal antigen-HLA complex. The interaction between CD8 and HLA class I molecules during antigen presentation would occur between CD8 molecules from the mature antigen receptor-positive T cell and the HLA class I molecules from the antigen-presenting cell. We do not know at present whether the CD1a intermolecular complexes occur between molecules from the same thymic cell or between molecules from different thymic cells. In the latter case it might explain why we do not observe intermolecular complexes on monoclonal cell proliferation. Moreover, the function of the CD1a intermolecular complexes could be to modulate positively or negatively the function of the molecules while they are associated in the complex. It is of interest that these complexes are observed on cortical thymocytes at a stage when the T cells are acquiring their antigen recognition structures that will recognize antigen in the context of the autologous HLA complex. Alternatively, the function of the CD1a intermolecular complexes could be to serve as a common matrix for the education of self-recognition. One should recall here that the genes coding for CD1 molecules have been postulated to be derived from a common ancestor to HLA class I or class II genes.²² It is thus of interest that the CD1 molecules, which can be considered remote members of the immunoglobulin supergene family,^{48,49} share with this family of recognition structures the characteristics to form associations.

Interactions between membrane-bound proteins play a significant role in a variety of cellular phenomena including the transduction of signals across membranes. Recently it was shown that the expression of high-affinity receptors for the interleukin 2 requires two distinct proteins from the cell surface that may be forming an intermolecular complex.⁵⁰⁻⁵² Thus the lack of intermolecular complexes may cause profound changes in the behavior of leukemic cells unable to upregulate or downregulate particular molecular functions appropriate to a discrete environment.

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Inhibition of Hemopoietic Growth Factor-Induced Proliferation by Adenosine Diphosphate-Ribosylation Inhibitors

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The effects of adenosine diphosphate (ADP) ribosylation inhibitors on hematopoietic growth factor-induced proliferation were examined. Significant inhibition of interleukin-3 (IL-3), colony-stimulating factor 1, and lung conditioned media-induced clonal agar growth of normal murine hematopoietic cells by 10 mmol/L nicotinamide (NAM), 10 mmol/L 3-aminobenzamide (3AB), and 5 mmol/L *N*¹-methylnicotinamide (1MN) was noted. Nicotinic acid, a related compound that does not inhibit ADP ribosylation, failed to inhibit the growth factor-mediated proliferation. NAM (10 mmol/L), 3AB (10 mmol/L), and 1MN (5 mmol/L) also prevented IL-3 and phorbol ester-stimulated ³H-thymidine incorporation into the IL-3-responsive FDC-P1 cell line. Exposure of FDC-P1 cells to 10 mmol/L NAM led to a significant decrease in nuclear poly-(ADP-ribose) levels. Exposure of FDC-P1 cells to 5 mmol/L 1MN did not affect

the interaction of the phorbol ester receptor, protein kinase-C (PK-C), with the cell membrane as determined by assay of phorbol ester binding in cytosol and membrane preparations. Nor did it affect the catalytic activity of PK-C as determined by assaying the *in vitro* phosphorylation of histone H1 by cytosolic kinase preparations from FDC-P1 as well as EL4 thymoma cells. 1MN markedly enhanced the inhibitory effects of phorbol esters on DNA synthesis of EL4 cells even at concentrations (1.25 mmol/L) that had no effects on DNA synthesis in the absence of phorbol esters. Our findings demonstrate that (a) active ADP ribosylation inhibitors interfere with growth factor-induced proliferation of murine hematopoietic cells and (b) the inhibition occurs at a step that follows the activation and translocation of PK-C and is more closely linked to DNA synthesis.

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THE PURIFICATION of the murine hematopoietic growth factors interleukin-3 (IL-3), colony-stimulating factor 1 (CSF-1), and granulocyte-macrophage colony-stimulating activity (GM-CSA) has permitted the evaluation of the intracellular events that mediate the proliferative response to these agents. Studies using an IL-3-responsive cell line (FDC-P1) have suggested that activation of protein kinase C (PK-C) occurs after exposure to IL-3.¹ The mechanism by which the interaction of IL-3 with its receptor and the subsequent activation of PK-C lead to cell proliferation is not known.

Adenosine diphosphate (ADP)-ribosylation of nuclear proteins, a reaction catalyzed by ADP-ribosyl transferase (ADPRT), plays an important role in the recovery from DNA damage induced by alkylating agents.^{2,3} Conflicting data exist regarding the possible role of this reaction in DNA replication. Some studies suggest that ADP ribosylation stimulates DNA synthesis, and others support the opposite conclusion.^{4,6} Experiments using ADPRT inhibitors have shown that these agents interfere with mitogen-induced activation (DNA synthesis) of human peripheral blood lymphocytes.^{6,7} These findings and the lack of a significant effect of these agents on the proliferation of the human T lymphoblastoid cell lines Molt 4 and CEM have been interpreted as

evidence supporting a role for ADP ribosylation in cell differentiation but not cell proliferation.⁷

To investigate the possible role of ADP ribosylation in the mediation of hematopoietic growth factor induced-proliferation, we studied the effects of three different ADPRT inhibitors (3-aminobenzamide [3AB], *N*¹-methylnicotinamide [1MN], and nicotinamide [NAM]) on CSF-1-, IL-3-, and GM-CSA-stimulated clonal agar growth of normal murine hematopoietic cells and on IL-3-induced proliferation of FDC-P1 cells. The changes in nuclear ADP-ribose levels in FDC-P1 cells in response to IL-3 in the presence and absence of the ADPRT inhibitors and the interaction of these agents with PK-C activation in FDC-P1 cells as well as in mouse thymoma EL4 cells were also examined. Our findings demonstrate a consistent inhibition of cellular proliferation by these agents and suggest a site of action at an intracellular step that occurs as a consequence of PK-C activation.

MATERIALS AND METHODS

Materials

NAM, 3AB, nicotinic acid (NA), 1MN, unlabeled phorbol esters, phosphatidyl serine (PS), 1,2-dioleoin, magnesium acetate, calcium chloride, adenosine triphosphate (ATP), ethyleneglycol tetracetic acid (EGTA), and EDTA were obtained from Sigma Chemical Co (St Louis). Methyl ³H-thymidine (³H-TdR, 100 µCi/mL) and [20-³H]phorbol-12,13-dibutyrate (PDB, 12.5 Ci/mmol in ethanol) were obtained from New England Nuclear (Boston). [γ-³²P]ATP, 0.15 to 1.0 Ci/mmol, was obtained from the Diabetes Research Center Core Laboratory at the University of Virginia where it was synthesized by the method of Johnson and Walseth.⁸ Purified IL-3 was kindly provided to us by Dr J. Ihle, National Cancer Institute, Frederick, MD. Purified CSF-1 was obtained from Dr R. Shadduck, Pittsburgh. Lung conditioned medium (LCM)⁹ was used as a source of GM-CSA. FDC-P1 cells developed by Dexter et al¹⁰ (provided by Dr J. Ihle, Frederick, MD) were grown at 37°C in RPMI 1640 (GIBCO, Grand Island, NY) with 10% fetal calf serum (FCS) (Hyclone, Logan, UT) and 25% conditioned medium from WEHI-3 cells (WEHI-CM) as a source of IL-3 under a 5% CO₂ atmosphere. Phorbol ester-sensitive EL4 cells¹¹ were maintained in RPMI 1640 with 5% heat-inactivated serum as previously described.¹² CBA-J

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mice (Jackson Laboratories, Bar Harbor, ME) were used as a source of bone marrow cells.

Methods

Bone marrow culture. Eight-week-old CBA-J mice were killed and their femurs and tibias were collected. Marrow from these bones was flushed into McCoy's medium containing 15% FCS. Cells (10^5 /mL) were plated into 35-mm plates containing different stimuli (IL-3, 25 U/mL; CSF-1, 400 U/mL; LCM 10% dilution) and in the presence and absence of 10 mmol/L NAM, 10 mmol/L 3AB, 10 mmol/L NA, or 5 mmol/L IMN.¹³ Each condition was set up in triplicate, and each experiment was done twice.

³H-TdR incorporation. FDC-P1 cells from exponentially growing cultures were washed four times in RPMI 1640 containing 10% FCS and used at a final concentration of 10^6 cells/mL. One hundred millimolar stock solutions of NAM, 3AB, NA, and IMN were prepared the day of the experiment. Dilutions of these reagents were made in 96-well microtiter plates (Costar, Cambridge, MA) to which 50 μ L of cells and maximally stimulatory concentrations of IL-3 (25 U/mL) or phorbol myristate acetate (PMA) (120 nmol/L) were added. Serial dilutions of IL-3 and PMA were also tested in the presence of fixed concentrations of the ADP ribosylation inhibitors in separate wells. The cells were incubated at 37°C and 5% CO₂, and 1 μ Ci of ³H-TdR was added 20 hours after the initiation of the cultures. Cells were harvested 4 to 6 hours later on a Skatron harvester (Sterling, VA), and ³H-TdR incorporation was determined by scintillation counting.

For ³H-TdR incorporation studies using EL4 cells, exponentially growing cells were resuspended at a concentration of 10^6 /mL in RPMI 1640 with 5% FCS, and 50 μ L was added to microtiter wells containing PMA (final concentration, 120 nmol/L), and/or the following concentrations of IMN: 5, 2.5, and 1.25 mmol/L. Incubation and ³H-TdR incorporation were as described for FDC-P1 cells. These experiments were performed three times ($n = 28$ per group) except for the groups with 1.25 mmol/L IMN, which are from a single experiment (12 replicates).

Proliferation in liquid cultures. FDC-P1 cells growing exponentially were seeded in growth media containing 10 mmol/L NAM, 10 mmol/L 3AB, 5 mmol/L IMN, 10 mmol/L NA, or no inhibitors. Cell counts as well as viability assessment using trypan blue dye exclusion were done daily.

Nuclear poly-(ADP-ribose) determinations. Exponentially growing FDC-P1 cells were washed four times in RPMI 1640 with 10% FCS and were resuspended to a final concentration of 7×10^7 cells/mL, and 1 mL of cells was added to 15-mL polypropylene tubes. IL-3 at 100 to 400 U/mL was added with and without 10 mmol/L NAM. A control group consisted of cells resuspended in RPMI 1640 plus 10% FCS. At different times after IL-3 addition, cells were centrifuged at 300 g for ten minutes, and the supernatant was discarded. The pellet was immediately resuspended in 25% trichloroacetic acid (TCA) and kept on ice for 30 minutes. Acid-insoluble material was collected by centrifugation at 600 g for ten minutes, and the pellets were washed once with TCA and twice with ether. Pellets were stored at -20°C before analysis. Pellets were solubilized in 1 mmol/L KOH and 10 mmol/L EDTA at 60°C for one hour. Guanidinium hydrochloride was added to a final concentration of 3 mmol/L, and the pH was adjusted to 9.1. Poly-(ADP-ribose) was then affinity purified by quantitative binding to dihydroboryl-BioRex 70 affinity resin. Poly-(ADP-ribose) was eluted from the affinity resin with 10 mmol/L HCl, and the polymer was then degraded to nucleosides by the action of snake venom phosphodiesterase and bacterial alkaline phosphatase. Fluorescent derivatives of the adenine nucleosides were formed by incubating the poly-(ADP-ribose)-derived nucleosides with chloroacetaldehyde at

60°C for four hours at pH 4.5. The ribose-containing fluorescent derivatives were affinity purified on PBA-60 (boronate affinity gel), and the derivatives were then separated by reverse-phase high-performance liquid chromatography. The poly-(ADP-ribose)-specific nucleoside derivative 1-*N*⁶-ethenoribosyladenosine was quantified by fluorescence detection as described previously.¹⁴

Phorbol ester (PDB) binding assay in FDC-P1 cells. FDC-P1 cells were washed three times in RPMI 1640 with 5% FCS, resuspended to a concentration of 1 to 2×10^6 /mL, and in three experiments, incubated in RPMI 1640 and 10% FCS for two hours (this incubation step was omitted in two other experiments). The inhibitor IMN at a final concentration of 5 mmol/L or vehicle was added to portions of the cells, and they were incubated at 37°C and 5% CO₂ for 30 minutes. They were washed with RPMI 1640 and ruptured in 2 mL of homogenization buffer (20 mmol/L Tris, pH 7.4, 0.33 mmol/L sucrose, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 10 μ g/mL leupeptin, and 50 μ mol/L 2-mercaptoethanol) with 20 strokes in a tight Dounce homogenizer. This preparation was centrifuged at 100,000 g for 45 minutes to yield cytosol (supernatant) and membrane (pellet) fractions. The pellet was resuspended in a volume of homogenization buffer equal to that of the cytosol and passed through a 23-gauge needle. Binding assays were done as previously described.¹² Briefly, cell fractions equivalent to 1.5 to 3×10^7 cells were incubated with 40 mmol/L [20-³H]PDB plus either a 100-fold excess of unlabeled PDB or vehicle (0.02% ethanol) for two to 18 hours at 4°C in the presence of bovine serum albumin at 1.2 mg/mL, 0.5 mmol/L CaCl₂, 75 mmol/L Mg acetate, and PS at 96 μ g/mL. Bound PDB was separated from free by filtration through glass fiber filters, and the radioactivity remaining on the filters was determined. Specific binding represents the difference between binding in the absence and that in the presence of competitor.

Protein kinase assay. Calcium/phospholipid-dependent protein kinase activity in crude cytosol preparations was assayed by determining the incorporation of [³²P] from [γ -³²P] ATP (2 to 4×10^6 cpm/tube) into histone H1 in the presence and absence of PS (40 μ g/mL) and diolein (1.6 μ g/mL). All of the incubations contained Mg acetate (5 mmol/L), CaCl₂ (60 nmol/L), and ATP (0.1 mmol/L).^{15,16} The reaction was stopped after three minutes by spotting an aliquot of the reaction mix onto phosphocellulose chromatography paper, and the radioactivity on the filters was determined after washing three times in 50 mmol/L NaCl and once in acetone. The effect of IMN on PK-C activity was determined by adding either the vehicle (deionized H₂O) or 5 mmol/L IMN to the cytosol preparation before the addition of the reaction mix.

Statistical methods. Analysis of variance with multiple comparisons (Duncan's test) was used. Results are expressed as means $\pm$ SEM. Statistical significance was accepted for $P < .05$. Statistical analysis of the phorbol ester-binding assay was done by using the Wilcoxon signed rank test.

RESULTS

Effect of ADPRT Inhibitors on Clonal Agar Growth of Bone Marrow Cells

Colony formation by hematopoietic cells in response to IL-3, LCM, and CSF-1 was significantly inhibited by 10 mmol/L NAM, 10 mmol/L 3AB, and 5 mmol/L IMN, whereas 10 mmol/L NA did not have an inhibitory effect (Fig 1). Inhibition of CSF-1 effects was more pronounced than the effect on IL-3-induced colony formation ($P < .05$). Macrophage colony formation in response to IL-3 was affected to a greater extent than granulocyte-containing colonies (of eight surviving colonies in the presence of 10 mmol/L NAM, seven were G-CFU and one was GM-CFU;

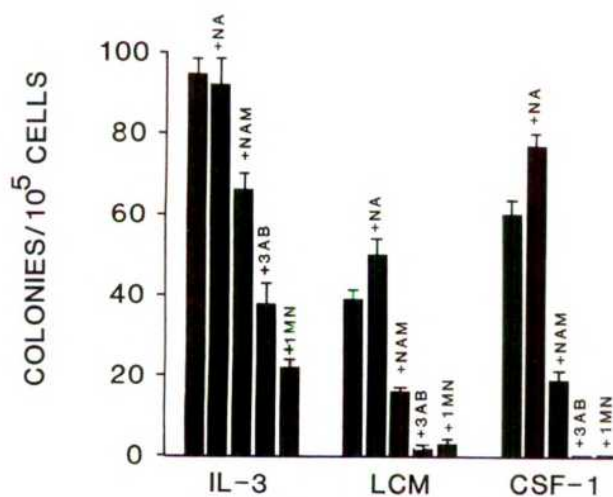


Fig 1. Effect of ADPRT inhibitors on IL-3-, GM-CSF-, and CSF-1-induced clonal agar growth of hematopoietic cells. Marrow cells were grown in agar in the presence of IL-3 at 25 U/mL, CSF-1 at 400 U/mL, or 10% LCM as a source of GM-CSF. Ten millimolar NAM, 10 mmol/L 3AB, or 5 mmol/L 1MN were added to experimental plates; sterile water (diluent) was added to control plates. Results expressed as means $\pm$ SE from two experiments ($n = 6$ per group).

this corresponds to a 95% inhibition of monocyte-containing colonies and a 13% inhibition of G-CFUs).

Effect of ADPRT Inhibitors on Thymidine Incorporation and Proliferation of FDC-P1 Cells

To eliminate problems in interpreting responses of mixed cell populations, we examined the effects of ADPRT inhibitors on IL-3-induced thymidine incorporation by FDC-P1 cells (a bone marrow-derived, IL-3-responsive cell line). Figure 2A shows the effects of different concentrations of NAM, 1MN, and 3AB on IL-3-induced ³H-TdR incorporation. NA at 10 mmol/L had no significant effect on IL-3-induced ³H-TdR incorporation of FDC-P1 cells ($P > .5$), whereas 10 mmol/L NAM, 10 mmol/L 3AB, and 5 mmol/L

1MN had a significant inhibitory effect ($P < .0001$, Fig 3A). This inhibitory effect was of the same magnitude over a wide range of IL-3 concentrations, which suggested that the inhibitors do not interfere with binding of IL-3 to its receptor (Fig 3A).

Since PK-C has been implicated in IL-3 action,¹ we examined the effects of the inhibitors on proliferation supported by the direct PK-C activator PMA. Inhibition of PMA-induced ³H-TdR incorporation by FDC-P1 cells at 24 hours was noted at the same concentrations tested in the experiments using IL-3 (Fig 2B). This inhibition was also noted over a wide range of PMA concentrations ($P < .0001$), thereby suggesting that this effect is not due to interference of these agents with the interaction between PMA and its proposed receptor, PK-C (Fig 3B). ³H-TdR incorporation in cultures containing NA and PMA was not any different from that of those containing PMA alone ($P > .5$).

The time course for this inhibitory effect on IL-3- and PMA-induced proliferation of FDC-P1 cells is shown in Fig 4. Ten millimolar NAM, 5 mmol/L 1MN, and 10 mmol/L 3AB inhibited the proliferation of the FDC-P1 cells in liquid cultures containing WEHI-CM as a source of IL-3. This effect became less evident with time, mostly due to a lack of cell proliferation in the control group by day 4, with all cultures reaching similar cell densities (Fig 4A). The inhibition by 1MN of PMA-induced proliferation of FDC-P1 cells was evident over a four-day period. Cell viability was the same in experimental and control cultures containing WEHI-CM during the first three days of culture, which suggested that the observed effects are not due to cell death.

Effect of ADPRT Inhibitors on Nuclear Poly-(ADP-Ribose) Levels

A significant decrease in the nuclear poly-(ADP-ribose) levels was detected within 20 minutes of exposure of FDC-P1 cells to 10 mmol/L NAM, thereby demonstrating that at the concentration tested these agents have one of their proposed biochemical effects, namely, inhibition of ADPRT (Fig 5).

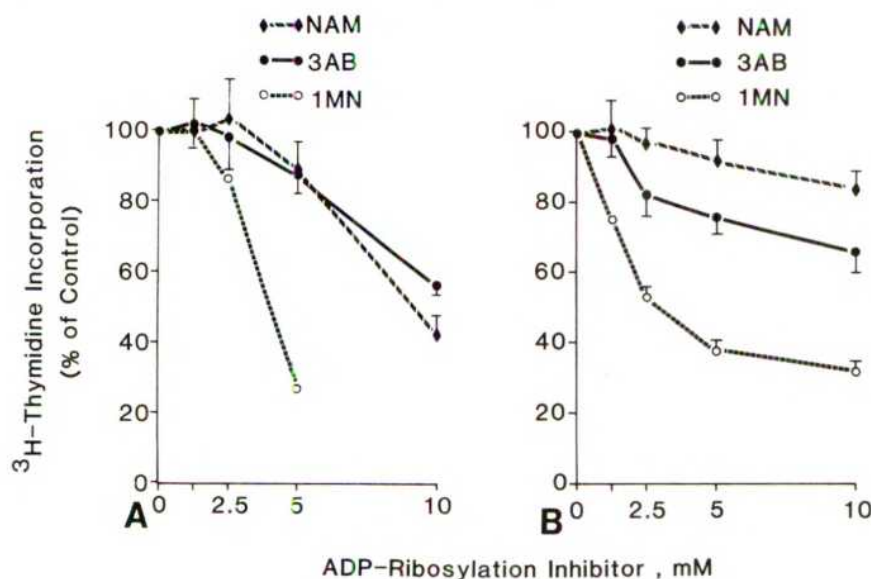


Fig 2. Dose-response curves for the effect of ADPRT inhibitors on (A) IL-3 (20 U/mL)- and (B) PMA (120 nmol/L)-induced thymidine incorporation into FDC-P1 cells. Results are shown on a semilogarithmic scale and expressed as the mean percentage of ³H-TdR incorporation (counts per minute) at 24 hours in cultures lacking the inhibitors. The curve for 1MN in A is from a single experiment; others are means of two separate experiments.

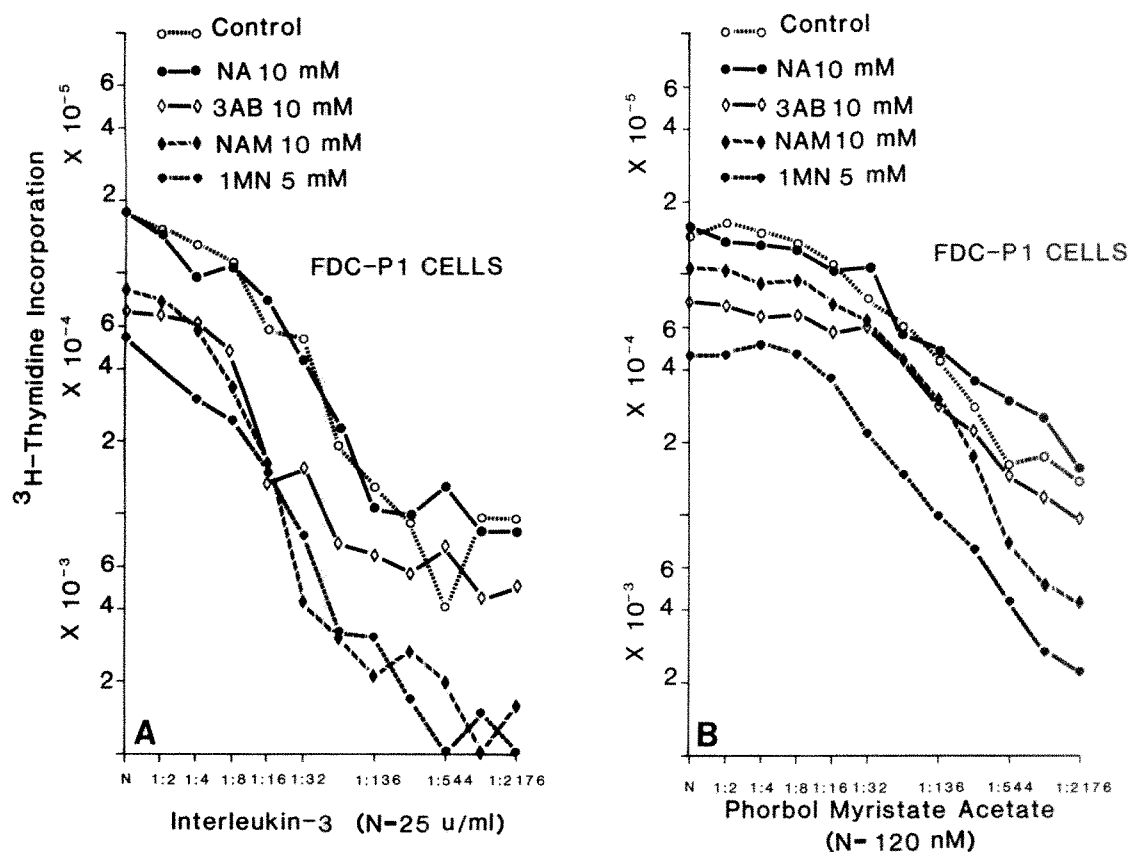


Fig 3. Effects of ADPRT inhibitors on (A) IL-3- and (B) PMA-induced ^3H -TdR incorporation of FDC-P1 cells. Serial dilutions of IL-3 and PMA were made in separate cultures containing fixed concentrations of tested substances: 10 mmol/L NAM, 10 mmol/L 3AB, 5 mmol/L 1MN, and 10 mmol/L NA. Results are mean ^3H -TdR incorporations at 24 hours from three experiments for A and two experiments for B.

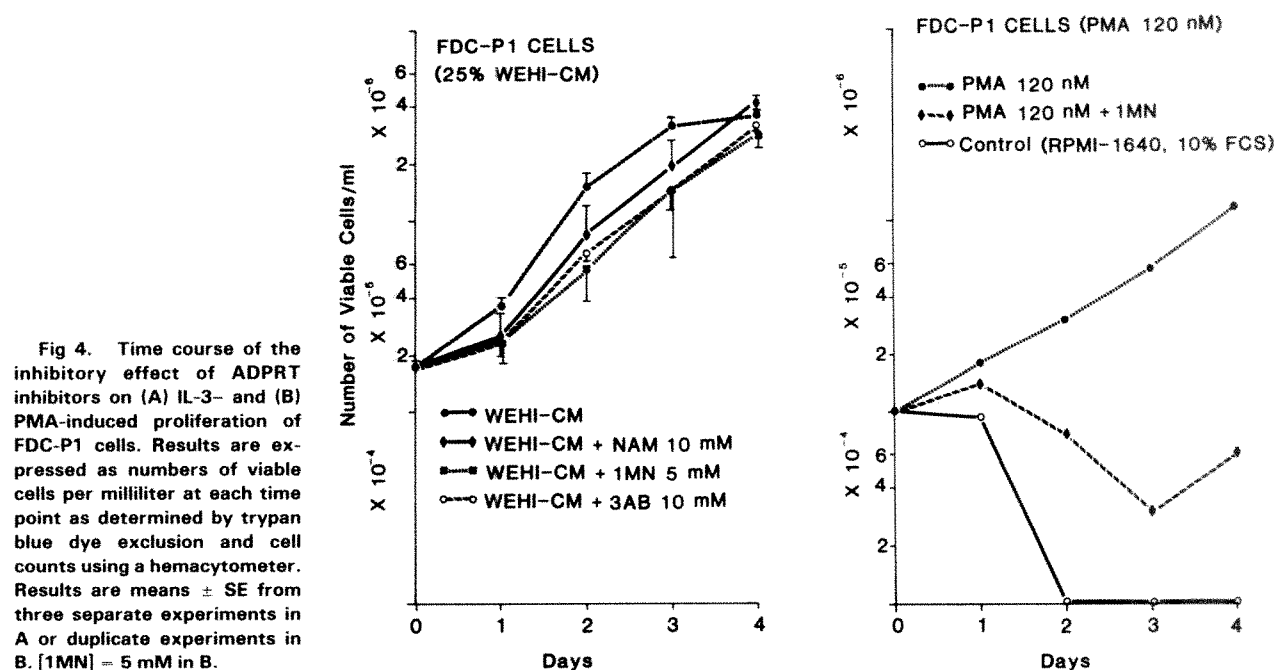


Fig 4. Time course of the inhibitory effect of ADPRT inhibitors on (A) IL-3- and (B) PMA-induced proliferation of FDC-P1 cells. Results are expressed as numbers of viable cells per milliliter at each time point as determined by trypan blue dye exclusion and cell counts using a hemacytometer. Results are means $\pm$ SE from three separate experiments in A or duplicate experiments in B. [1MN] = 5 mM in B.

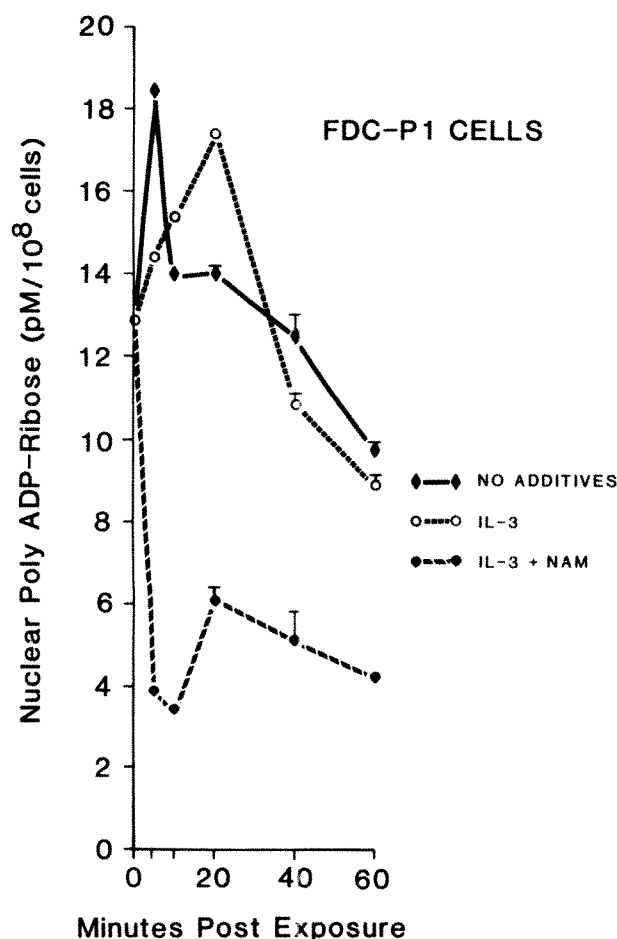


Fig 5. Effects of ADPRT inhibitors on nuclear poly-(ADP-ribose) levels in FDC-P1 cells. [IL-3] = 100 to 400 U/mL; 10 mmol/L NAM was used.

Effect of ADPRT Inhibitors on Subcellular Location and Activity of PK-C

Since the ³H-TdR incorporation experiments suggested that these ADPRT inhibitors specifically affect the PK-C pathway in FDC-P1 cells, we next attempted to determine the step in this pathway that is inhibited by these agents. Exposure of FDC-P1 cells to 1MN did not lead to changes in the cytosol/membrane PDB binding ratio, thereby suggesting that these agents do not interfere with PK-C/phospholipid interaction (Fig 6). Five millimolar 1MN did not alter the phorbol ester (PDB)-induced changes in the cytosolic/membrane PDB binding ratio (two separate experiments, data not shown). Nor did cytosols recovered from FDC-P1 cells that had been exposed to 1MN at 5 mmol/L for 30 minutes differ from control cytosols in their total kinase or calcium/phospholipid-dependent kinase activity (PK-C) as determined by *in vitro* phosphorylation of histone H1 (Table 1). The presence of 5 mmol/L 1MN in the reaction mix did not affect the calcium/PS-dependent kinase activity present in crude cytosolic preparations of FDC-P1 or EL4 cells (Table 1).

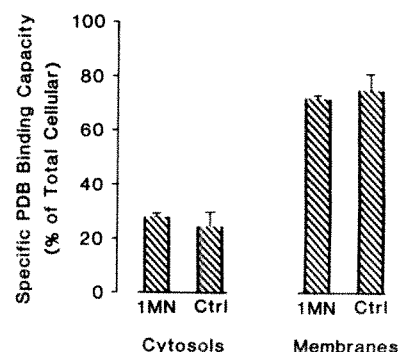


Fig 6. Effects of ADPRT inhibitors on subcellular location of PK-C. Results are means $\pm$ SE from five separate experiments. Values ranged from 3.1×10^3 to 11.1×10^3 cpm/ 10^6 cells for total cytosolic binding and from 0.9×10^3 to 3.0×10^3 for nonspecific cytosolic binding. Membrane binding ranged from 10.3×10^3 to 19.0×10^3 cpm/ 10^6 cells for total and from 1.4×10^3 to 5.7×10^3 cpm/ 10^6 cells for nonspecific binding.

Effect of 1MN on EL4 Cell Proliferation

The evidence presented thus far suggests that these agents inhibit the proliferation of FDC-P1 cells by blocking the PK-C pathway at a step that follows activation of PK-C. To determine whether this effect was specific for the PK-C pathway or for cell proliferation, we investigated the effects of 1MN on PMA-induced effects on EL4 cells. Proliferation of these mouse thymoma cells is inhibited by PMA; this effect is also accompanied by increased IL-2 production.¹¹ We found that 1.25 mmol/L 1MN had no effect on thymidine incorporation of exponentially growing EL4 cells ($P > .05$). In the presence of PMA, 1.25 mmol/L 1MN markedly enhanced the inhibitory effect of PMA on ³H-TdR incorporation (Fig 7). Five millimolar 1MN inhibited ³H-TdR incorporation by EL4 cells to a lesser degree than in FDC-P1 cells.

DISCUSSION

The results from the bone marrow experiments reported herein demonstrate that ADP ribosylation inhibitors signifi-

Table 1. Effect of 1 MN on Calcium/Phospholipid-Dependent Kinase Activity

Sample	A (+PS)	B (-PS)	C (A - B)
FDC-P1 cytosol	4.5 $\pm$ 0.02	3.3 $\pm$ 0.01	1.2 $\pm$ 0.12
FDC-P1 cytosol + 1MN	4.5 $\pm$ 0.3	3.4 $\pm$ 0.15	1.1 $\pm$ 0.32
FDC-P1 cytosol	4.9 $\pm$ 0.46	3.1 $\pm$ 0.21	1.8 $\pm$ 0.61
FDC-P1/1MN cytosol	4.5 $\pm$ 0.43	2.8 $\pm$ 0.37	1.7 $\pm$ 0.18
EL4 cytosol	4.7 $\pm$ 0.43	2.7 $\pm$ 0.40	2.0 $\pm$ 0.25
EL4 cytosol + 1MN	4.8 $\pm$ 0.42	2.9 $\pm$ 0.40	1.9 $\pm$ 0.12

Results are counts per minute per 10^5 cells (mean $\pm$ SEM $\times 10^3$). FDC-P1 + 1MN: 5 mmol/L 1MN added to FDC-P1 cytosol during assay. FDC-P1/1MN cytosol: cytosol obtained from cells exposed to 5 mmol/L 1MN for 30 minutes. EL4 cytosol + 1MN: 5 mmol/L 1MN added to EL4 cytosol in the assay. Results shown are means $\pm$ SE from three separate experiments for the FDC-P1 experiments and from four separate experiments involving EL4 cells. Column A, total kinase activity in samples; column B, kinase activity in the absence of phospholipids; column C, value obtained from subtracting B from A—Ca/phospholipid-dependent kinase activity.

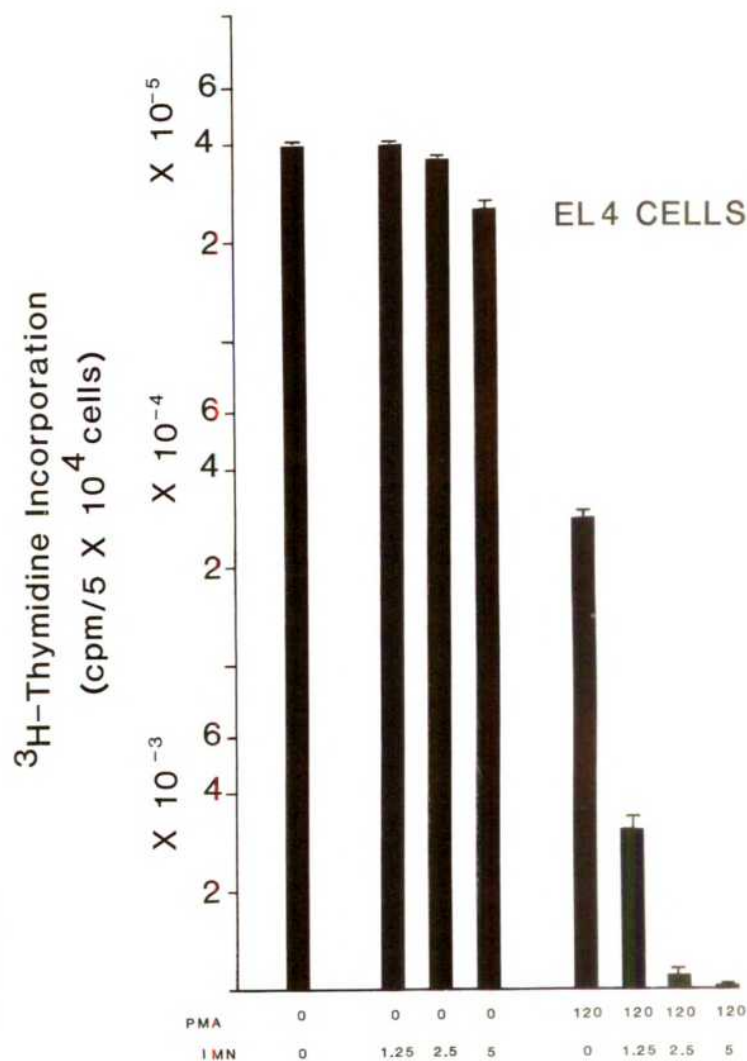


Fig. 7. Effects of 1MN on ³H-TdR incorporation into EL4 cells. Results are expressed as means $\pm$ SE from three separate experiments ($n = 28$ per group). 1MN (millimolar) and PMA (nanomolar) were added at the same time, and ³H-TdR incorporation was determined 24 hours later. The control group consisted of EL4 cells exposed to RPMI 1640 plus 5% FCS.

cantly interfere with IL-3-, CSF-1-, and GM-CSA-induced clonal agar proliferation of murine hematopoietic cells. Francis et al investigated the effects of three different ADPRT inhibitors (5-methylnicotinamide, 3AB, and 3-methoxybenzamide) on clonal agar growth of human marrow cells in the presence of a leukocyte feeder layer as a stimulus and found that these agents preferentially inhibited the growth of macrophage-granulocyte and pure macrophage clones over that of granulocyte colonies.¹⁷ On the basis of these results the authors suggested that ADP ribosylation is involved in macrophage differentiation. Dexter et al investigated the effects of two different inhibitors of ADP ribosylation (benzylaminododecylguanine hydrochloride and *p*-methoxybenzylaminodecamethylene guanidine sulphate) on murine hematopoiesis by using long-term marrow cultures.¹⁸ They found that these agents decreased the number of WEHI-CM-responsive colony-forming cells to a lesser extent than their effect on the number of CSF-1-responsive cells.¹⁸ We found a similar hierarchy of inhibition with the ADPRT inhibitors we studied.

The inhibition by ADPRT inhibitors of IL-3- and PMA-

induced proliferation of FDC-P1 cells suggests that these agents interfere with PK-C activation or with an intracellular event that occurs as a consequence of PK-C activation. The lack of an effect on the subcellular location or catalytic activity of PK-C suggests that the inhibition is at a step that follows the activation of PK-C.

Previous studies by Singh et al using mouse embryo fibroblasts (C3H10T1/2) and human monocytes provide further evidence for an interaction between PMA-induced effects and ADP ribosylation.¹⁹⁻²¹ These studies suggested a role for ADP ribosylation of nuclear proteins in the modulation of gene expression induced by PMA and suggested that an oxidative burst is an intermediate step in this effect. An increase in poly-(ADP-ribose) levels and DNA breaks occurred in human monocytes within the first hour of exposure to PMA; these effects were blocked by antioxidants.²¹ It has been proposed that stimulation of phospholipase A₂ by PMA, as a consequence of PK-C activation, leads to the stimulation of arachidonic acid metabolism and increased formation of hydroperoxyarachidonic acid intermediates.²² These unstable products decay spontaneously to

the corresponding hydroxyl derivatives releasing active oxygen, the latter being highly efficient in inducing DNA breaks.²² Studies using P388D₁ murine macrophages have shown that inhibition of DNA synthesis and activation of ADPRT occurs during the first hour of exposure to 40 μ mol/L H₂O₂, probably as a consequence of oxidant-induced single-strand DNA breaks.²³

It is possible that PMA induces a similar oxidative burst in EL4 cells. Copper complexes with superoxide dismutase mimetic activity can inhibit phorbol ester-induced interleukin-2 production; however, it has not been demonstrated that the inhibitory activity of these compounds is due to their dismutase mimetic activity.²⁴ Studies using human peripheral blood lymphocytes suggest that rejoining of DNA breaks is required for proceeding with mitogen-stimulated DNA synthesis and that ADPRT plays a significant role in this DNA repair.²⁵ Therefore, it is possible that the 1MN-induced enhancement of the inhibitory effects of PMA on DNA synthesis in EL4 cells is due to the role of ADPRT in repairing oxidant-induced DNA breaks and the requirement for this DNA repair before resuming DNA synthesis.

One cannot conclude that the observed effects of the ADPRT inhibitors reported herein are entirely or primarily due to their effect on nuclear ADP ribosylation. These agents inhibit other nonnuclear ADP ribosylation reactions, among which there are reactions that have been associated with the adenylate cyclase system and mitochondrial calcium efflux.²⁶⁻²⁸ NAM at the concentration studied (10 mmol/L) had been reported to lower the intracellular ATP levels.²⁹ 3AB also inhibits de novo synthesis of DNA precursors in lymphoblast cells, although this effect does not seem to be responsible for its cell replication effects as suggested by similar sensitivity to this agent of lymphoblast cells proficient or deficient in this pathway.³⁰ The most potent inhibitor of hematopoietic proliferation was 1MN, which is the least potent inhibitor of ADPRT activity in vitro among those

agents studied.³¹ 3AB at 2.5 mmol/L had minimal, if any, inhibitory effects on IL-3-induced ³H-TdR incorporation of FDC-P1 cells. This concentration of 3AB has previously been shown to inhibit ADPRT activity in permeabilized cells.²⁵ Still, due to differences in intracellular metabolism between these drugs, one cannot equate in vitro potency with their effects on intact cells. The decrease in nuclear ADP-ribose concentrations after treatment of FDC-P1 cells with NAM is consistent with inhibition of nuclear ADPRT.

The lack of a demonstrable quantitative increase in nuclear poly-(ADP-ribose) levels in FDC-P1 cells after exposure to IL-3 does not necessarily imply that ADP ribosylation is not involved in the mediation of IL-3-induced proliferation. It is possible that qualitative rather than quantitative changes in nuclear ADP ribosylation occur in response to IL-3. Nuclear ADP ribosylation may be involved in cell proliferation in one of several possible ways. Histones are substrates for this reaction, and in vitro studies have shown that inhibition of DNA ligase activity by histones is abolished by ADP ribosylation.³² As mentioned earlier, rejoining of DNA strand breaks in resting lymphocytes has been shown to be an early event in their proliferation in response to mitogens, and ADPRT has been shown to participate in this process.²⁵ In vitro studies using isolated rat pancreatic polynucleosomes have shown a direct correlation between polynucleosome relaxation, histone H1 ADP ribosylation, and activation of DNA polymerase activity.³³ Finally, DNA topoisomerase I, an enzyme that has been implicated in the regulation of DNA replication, is ADP ribosylated in vitro by ADPRT.³⁴ In vitro phosphorylation of topoisomerase II by PK-C has been reported, as has phosphorylation of topoisomerase I by other kinases.^{35,36} It is possible that the interaction between the phorbol ester-induced effects and the ADPRT inhibitors' effects occurs at the level of DNA topoisomerase activity.

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Exposure to γ -Irradiation Increases Phorbol Myristate Acetate-Induced H_2O_2 Production in Human Macrophages

By Elaine K. Gallin and Spencer W. Green

Cell number, protein, and phorbol myristate acetate (PMA)-induced H_2O_2 production were measured in cultured human peripheral blood monocytes for six days after exposure to varying doses of γ -radiation. Both the number of adherent cells and the protein per dish decreased with increasing radiation doses. The dose of radiation decreasing the number of adherent cells by 37% on days 4 and 6 postirradiation was 29 Gy. Four hours postirradiation there was a small decrease in PMA-induced H_2O_2 production for doses of 7.5 Gy or greater; levels returned to normal by eight hours and increased at 24 hours postirradiation. By day 4 postirradiation significant increases in PMA-induced H_2O_2 production were noted at all radiation doses (2.5 to 50 Gy). This increase was not due to a shift in the PMA dose-response curve, a change in the time course of the

PMA response, or an effect of decreased cell density on the assay system. Superoxide levels were not significantly changed in cells exposed to 20 Gy. Catalase, glutathione peroxidase, and superoxide dismutase levels also were unchanged. Culturing irradiated cells with γ -interferon increased PMA-induced H_2O_2 release, which indicated that irradiated cells retained their capacity to respond to γ -interferon. These data demonstrate that irradiation affects the PMA-induced H_2O_2 production of human monocytes in a time- and dose-dependent manner. An increase in the release of reactive oxygen intermediates by the macrophage may play a role in enhancing the deleterious effects of radiation in vivo.

This is a US government work. There are no restrictions on its use.

MACROPHAGES, although relatively insensitive to the effects of radiation compared with lymphocytes, exhibit time-dependent changes in cell function and survival after exposure to ionizing radiation in doses of 7.5 to 20 Gy. At four days postirradiation, mouse peritoneal macrophages exhibit a decrease in Fc-mediated phagocytosis¹ and a decrease in Ia expression.² The acid phosphatase activity of mouse peritoneal macrophages exposed to 5 Gy or more is unaffected three hours postirradiation but increases by 24 hours.³ In human peripheral blood monocytes, exposure to 25 or 50 Gy decreased the 6-week survival of blood monocytes in culture and diminished the rate of microbial killing of the surviving cells.⁴ In this paper, we show that exposure of human peripheral blood monocytes to γ -radiation decreases the number and size of adherent cells within 48 hours postirradiation. However, the surviving cells exhibit an increase in PMA-induced H_2O_2 production that is evident 24 hours postirradiation and maintained throughout the six-day culture period.

The production of H_2O_2 by macrophages is associated with both extracellular cytotoxicity and inhibition of intracellular pathogens.^{5,6} Thus, the ability of macrophages to produce H_2O_2 in response to phorbol esters or particulate stimuli is associated with cell activation. However, H_2O_2 and other

reactive oxygen intermediates, in addition to protecting against pathogens, can themselves produce extensive tissue damage.^{7,8} It is possible that tissue damage postirradiation is due in part to augmentation of the production of H_2O_2 by the tissue macrophage.

MATERIALS AND METHODS

Cells. Human monocytes were isolated from leukopaks supplied from the National Institutes of Health blood bank by using lymphocyte separation media (Litton Bionetics, Bethesda, MD). All blood donors were advised of procedures and attendant risks in accordance with institutional guidelines. The cellular band containing lymphocytes and monocytes was washed with phosphate-buffered saline without calcium and magnesium (PBS/0 Ca,Mg) and centrifuged through fetal calf serum (FCS) (Sterile Systems, Logan, UT) at 100 g for ten minutes. The cells were resuspended in PBS/0 Ca,Mg containing 10% FCS, and placed on Percoll gradients (Pharmacia Fine Chemicals, Piscataway, NJ). The gradients were centrifuged at 1,000 g for 20 minutes at 4°C; the monocyte band was isolated and washed twice with PBS/0 Ca,Mg. The monocytes were resuspended in tissue culture medium consisting of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10 U/mL of penicillin (Difco, Detroit), 10 μ g/mL streptomycin (Difco), 0.03% L-glutamine (Sigma Chemical Co, St Louis), and 10% heat-inactivated AB human serum (Bio-tee, Boston). The mononuclear cell layer obtained after Percoll isolation contained approximately 90% monocytes as assessed by esterase staining. In most experiments, cells were plated on cluster-6 tissue culture dishes at concentrations of 1 to 2×10^6 cells/well. Cells were allowed to adhere for two hours and then were washed twice to remove nonadherent cells and refed with complete tissue culture medium. In some instances cells were placed in Teflon jars after isolation and were cultured for varying periods of time before plating and irradiating.

Cell number and protein. Cell number was determined on adherent populations of cells by counting the number of adherent cells in three separate 250 $\times$ fields of the culture dish with inverted phase microscopy. The total number of cells in the dish was then determined from the area of the microscope field and the surface area of the dish. For protein measurements cells were washed three times with PBS to remove serum proteins, and 1 mL of 0.2% Triton X-100 (New England Nuclear, Boston) was added to the dishes. Cell lysates were measured by using the Bradford method (Bio-Rad, Richmond, CA). Bovine serum albumin (Bio-Rad) was used as the protein standard. Radiation does not result in any measurable serum

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protein sticking to the surface of the culture dishes. Protein per cell was obtained by dividing the total protein by the number of cells. All experiments were done in triplicate or greater.

Radiation. Cells were irradiated at varying times after isolation bilaterally by using a cobalt 60 γ -radiation source at a constant dose rate of 5 Gy/min.

H_2O_2 measurements. H_2O_2 release was assayed by measuring the decrease in fluorescence associated with the oxidation of scopoletin by H_2O_2 .⁹ Adherent cells were washed twice with PBS/Ca, Mg and 2 mL of reaction mixture containing PBS, 10 mmol/L glucose, and 10 to 80 nmol/L scopoletin (Sigma), and 6 purpurogallin units of horseradish peroxidase/mL (Sigma) at pH 7.1 was added to each well. The stimulus, phorbol myristate acetate (PMA) (Consolidated Midland, Brewster, NY) at a concentration of 100 ng/mL (0.01% final dimethyl sulfoxide concentration), was added to some of the wells. In a few studies 1 mmol/L sodium azide was also added to the reaction mixture. The cells were incubated with the reaction mixture at 37°C for one hour, after which the solution was removed from the cells. The fluorescence of each sample was measured by exciting at 350 nm and recording at 460 nm. Reaction mixture not exposed to cells was used as the control. Scopoletin levels was adjusted so that the scopoletin concentration was not the limiting factor, i.e., the fluorescence levels were never reduced by more than 60%. H_2O_2 levels were calculated by comparing the fluorescence decrease produced by the cells with the fluorescence decrease caused by known amounts of ethyl peroxide (Polysciences, Warrington, PA).

Superoxide anion measurements. Superoxide was assayed by measuring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c.¹⁰ Cells were washed twice with Krebs-Ringer phosphate-containing glucose (KRPBG), and then 2 mL of reaction mixture (KRPBG containing 50 μ mol/L ferricytochrome c [Sigma] with or without 0.1 mg/mL of SOD) was added to each dish. PMA was added at 100 ng/mL to the dishes. Dishes were incubated at 37°C for one hour, at which time the absorbance of the supernatant at 550 nm was measured. The value of the absorbance with SOD was subtracted from the value of the absorbance without SOD to determine superoxide levels. Superoxide levels were computed by using an extinction coefficient of 21,000.

SOD, glutathione peroxidase, and catalase measurements. For SOD and glutathione peroxidase measurements cells were rinsed twice with PBS and exposed to 0.2% Triton X-100 for 30 minutes at room temperature. Cell lysates were assayed for SOD activity by using the method of McCord and Fridovich.¹¹ This method assays the inhibition by SOD of the reduction of ferricytochrome c by superoxide. Xanthine and xanthine oxidase were used to generate the superoxide. Glutathione peroxidase was measured on 5 to 10 $\times 10^6$ cells/mL according to the method of Paglia and Valentine.¹² Catalase was measured on cell fractions obtained by scraping adherent cells and sonicating them on ice for 40 seconds. Cell sonicates were then assayed for their ability to break down H_2O_2 by following the destruction of H_2O_2 spectrophotometrically at 230 nm.¹³ All enzyme assays were done at room temperature.

γ -Interferon. In a few studies cells were cultured in the presence of recombinant human γ -interferon (γ IFN) (AmGen, Thousand

Oaks, CA) for varying periods of time before assaying PMA-induced H_2O_2 release.

RESULTS

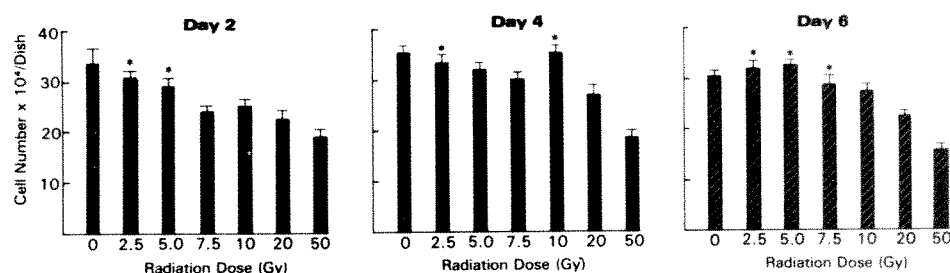
Effect of radiation on cell number and cell size. Figure 1 shows the data from one representative experiment examining the effects of radiation on the number of adherent macrophages in culture on days 2, 4, and 6 postirradiation. Exposure to 2.5 Gy did not significantly decrease the cell number on any of the days assayed. However, with increasing doses of radiation, the cell number decreased significantly. The data from days 4 and 6 from this experiment and others are plotted in Fig. 2. The data are expressed as a percentage of control (nonirradiated) values. The line drawn through the points represents a least-squares fit to a single exponential function. The D_0 (dose of radiation decreasing the cell number by 37%) determined from this plot was 29 Gy.

Total protein per dish, shown in Table 1, also decreased in a dose- and time-dependent manner in irradiated cells. By days 4 and 6 postirradiation, protein levels decreased by 50% or more in dishes exposed to 20 and 50 Gy. The protein-per-cell values calculated from the protein and cell number data indicated that the surviving cells were smaller than control cells. That is, the protein-per-cell levels decreased by 18% to 37% on days 4 and 6 postirradiation.

H_2O_2 production. PMA-induced H_2O_2 release was measured in both control and irradiated cells at varying times postirradiation. Data from a representative experiment are shown in Fig 3A. As previously reported by Nakagawara et al.,⁹ the levels of PMA-induced H_2O_2 released by control (nonirradiated) macrophages decreased with time in culture. With the exception of the 5-Gy, two-day data point, all radiation doses tested produced a significant increase in H_2O_2 release compared with the control. With increasing radiation doses the decrease in H_2O_2 production, which occurred in control cells with time in culture, was abrogated. Since our data demonstrated that radiation produces a decrease in the protein per cell, the increase in H_2O_2 expressed in terms of protein could result from cells with a reduced protein content producing the same amount of H_2O_2 . To determine whether this was the case, the data were expressed in terms of cell number rather than milligrams of protein. The results are shown in Fig 3B. The data are qualitatively similar and show a dose- and time-dependent increase in H_2O_2 release after irradiation.

Table 2 summarizes a number of experiments measuring H_2O_2 levels at additional time points. Because of the variability in the H_2O_2 production between different groups of cells,

Fig 1. Dose response of effect of radiation on number of cells per dish at 2, 4, and 6 days postirradiation. Cells were irradiated one day after isolation. Each point is the average of four measurements $\pm$ SE. *, Groups not significantly different from control ($P > .01$).



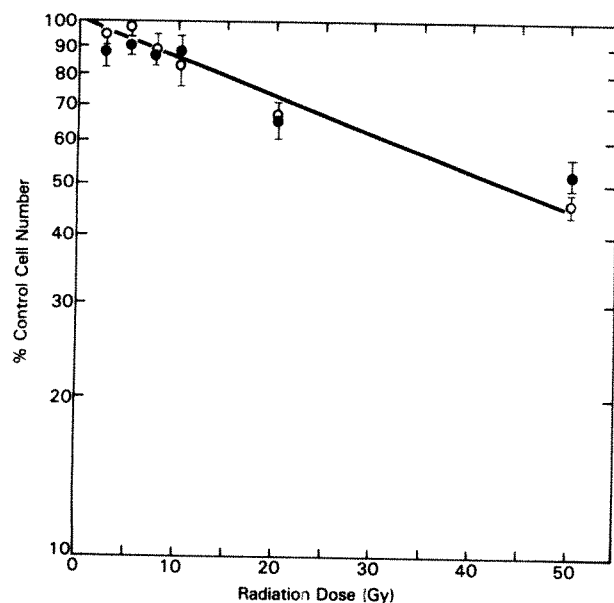


Fig 2. Dose response of effect of radiation on the number of adherent cells in culture at four (●) and six (○) days postirradiation. Cells were irradiated one day after isolation. Each data point represents the average $\pm$ SE of three separate studies, each done in triplicate or greater. The line drawn through the data is a least-squares fit ($R^2 = .997$).

the data are expressed as a percentage of control values. At four hours postirradiation there was actually a small but significant decrease in PMA-induced H_2O_2 production for cells exposed to 7.5 Gy or greater. Eight hours postirradiation, H_2O_2 release in cells irradiated with 10 Gy or more was still slightly reduced. It should be noted that the H_2O_2 levels obtained at four and eight hours were probably reduced by the myeloperoxidase activity present in monocytes 24 to 48 hours after isolation.⁹ However, the data from cells cultured for 48 hours or longer (24 hours or longer postirradiation) was not influenced by myeloperoxidase activity (see the following section on sodium azide effects). At 24 hours postirradiation, exposure to 5 Gy produced only a 16% decrease in the protein per dish (Table 1) but a 45% increase in H_2O_2 . This increase was even greater on day 2 postirradiation, was maintained on day 4, and increased further on day 6 postirradiation.

In the aforementioned studies, cells were exposed to 100 ng/mL PMA. To ensure that the dose of PMA was supra-maximal for both control and irradiated cells, PMA dose-

response curves were obtained for both control and irradiated (20 Gy) cells four days postirradiation. As shown in Fig 4, the dose-response curves for control and irradiated cells differed only in the magnitude of the H_2O_2 released.

The preceding studies were done on human peripheral blood monocytes that were irradiated 24 hours after isolation. To determine whether similar changes occur in more mature macrophages, cells were cultured in Teflon dishes for six to 14 days, plated on tissue culture dishes, washed to remove nonadherent cells, and irradiated with 20 Gy. PMA-induced H_2O_2 was measured on days 2 and 4 postirradiation. These studies indicated that mature human macrophages also exhibit an increase in PMA-induced H_2O_2 release after exposure to γ -radiation.

H_2O_2 measurements at a constant protein content per dish. Since the radiation doses (20 and 50 Gy) that produce the largest increase in H_2O_2 production on days 4 and 6 also decreased the cell protein content and cell number by 50% or greater, experiments were done to investigate the effects of cell density on PMA-induced H_2O_2 release. Human monocytes were plated at varying densities, and cultured for 1, 4, or 6 days; then the PMA-induced H_2O_2 release was assayed. In two out of three experiments, H_2O_2 levels increased as the protein per dish decreased below 15 μ g/dish. To ensure that the increases in H_2O_2 were due solely to an effect of radiation and not simply a decrease in the protein per dish, a series of experiments were done in which monocytes were plated at varying densities before irradiation to compensate for the decrease in cell number and protein content after irradiation. The open circles in Fig 5 plot the data from cells assayed four days postirradiation. These data represent the average of three different experiments expressed as a percentage of control H_2O_2 release. It is evident that, even under conditions in which the protein per dish did not vary, radiation produced significant increases in H_2O_2 production. The line drawn through the points represents a least-squares fit to a bioexponential function. The closed circles in Fig 5 represent the data from Table 2 that were obtained from studies in which irradiated cells were plated at the same density as control cells so that the protein per dish was less than control values at the time of the measurements (Table 1). With the exception of the 20-Gy and 50-Gy points, the two sets of data are quite similar, indicating that the increase in H_2O_2 release by irradiated cells is not an artifact of decreased cell density.

To determine whether the time course of PMA-induced

Table 1. Time Course of Change in Protein per Dish Postirradiation

Time Postirradiation (h)	Radiation Dose (Gy)						n*
	2.5	5.0	7.5	10	20	50	
4	115 $\pm$ 7	109 $\pm$ 7	98 $\pm$ 7	102 $\pm$ 9	89 $\pm$ 10	95 $\pm$ 7	12
8	95 $\pm$ 5	92 $\pm$ 5	80 $\pm$ 5	93 $\pm$ 7	93 $\pm$ 5	100 $\pm$ 6	12
24	94 $\pm$ 5	83 $\pm$ 4	83 $\pm$ 5	80 $\pm$ 6	72 $\pm$ 11	86 $\pm$ 5	15
48	86 $\pm$ 5	91 $\pm$ 4	85 $\pm$ 4	79 $\pm$ 5	72 $\pm$ 8	54 $\pm$ 3	12
96	77 $\pm$ 4	71 $\pm$ 3	70 $\pm$ 2	67 $\pm$ 3	52 $\pm$ 3	35 $\pm$ 2	12
144	87 $\pm$ 4	81 $\pm$ 3	70 $\pm$ 3	68 $\pm$ 6	46 $\pm$ 4	43 $\pm$ 4	12

Data are expressed as a percentage of control (unirradiated) values. Human mononuclear cells were irradiated 24 hours after isolation.

*Number of data points for each time point.

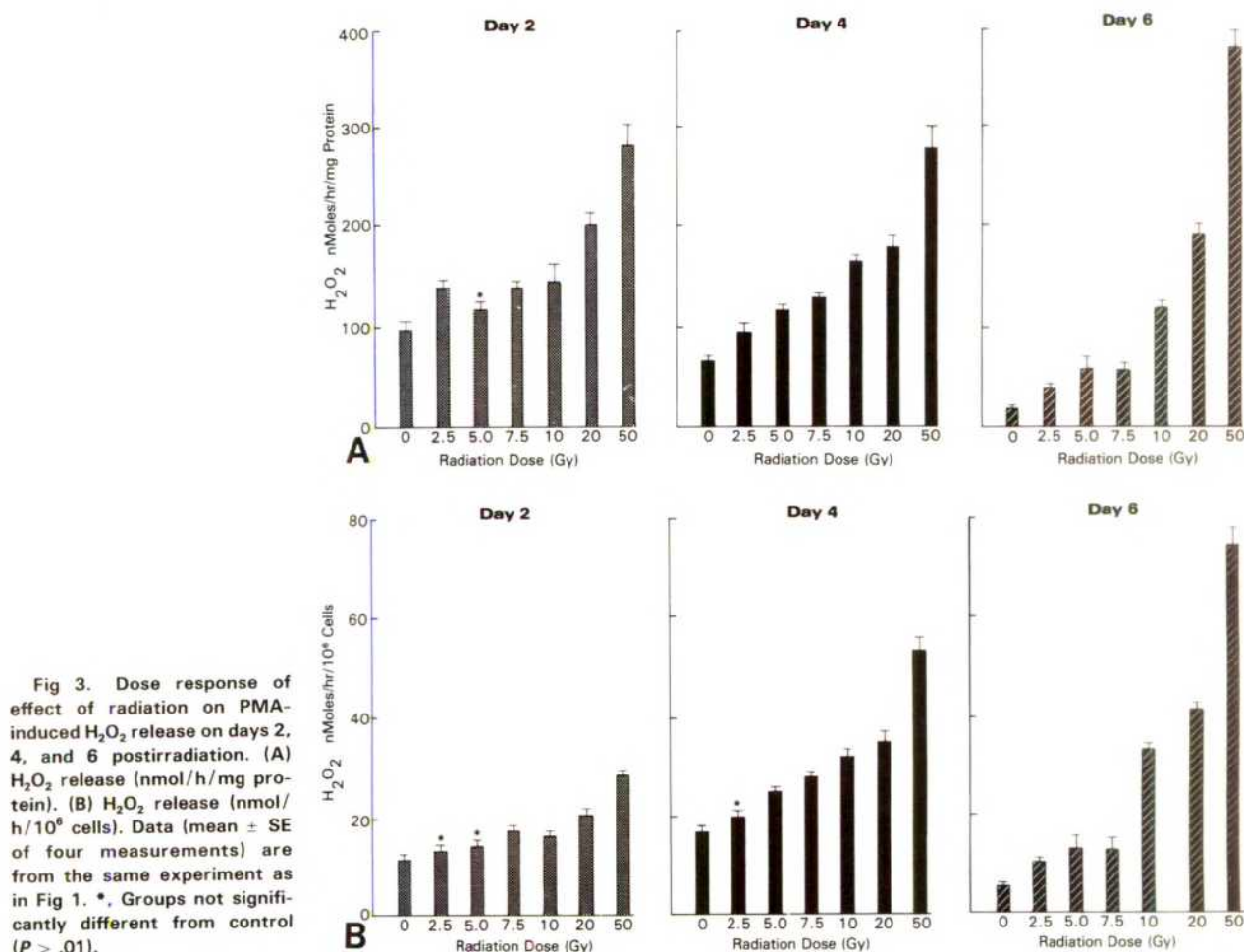


Fig 3. Dose response of effect of radiation on PMA-induced H_2O_2 release on days 2, 4, and 6 postirradiation. (A) H_2O_2 release (nmol/h/mg protein). (B) H_2O_2 release (nmol/h/ 10^6 cells). Data (mean $\pm$ SE of four measurements) are from the same experiment as in Fig 1. *, Groups not significantly different from control ($P > .01$).

H_2O_2 release in irradiated and control cells was similar, supernatants from PMA-treated wells were harvested at various times after the addition of PMA (100 ng/mL). Figure 6 shows the data from a typical experiment in which control and irradiated (20 Gy) cells were examined. It is evident that the time course of H_2O_2 release in both groups of cells was similar even though the total H_2O_2 released by the irradiated cells was doubled. In this experiment irradiated cells were plated at a higher density so that the protein per dish was similar in both groups of cells.

Effect of sodium azide. H_2O_2 release by freshly isolated human monocytes that contain high levels of myeloperoxidase

is increased fourfold by the addition of sodium azide (which inhibits myeloperoxidase) to the reaction mixture.⁹ Myeloperoxidase levels decrease in monocytes during the first two days in culture so that sodium azide has little effect on H_2O_2 production after day 2.⁹ To confirm that the H_2O_2 levels measured in these studies were maximal (ie, that they could not be further enhanced by myeloperoxidase inhibitors), several studies were done in the presence of 1 mmol/L sodium azide. The addition of sodium azide to cultures ten minutes previous to and during exposure to PMA increased (3.8-fold) H_2O_2 levels in macrophages assayed four hours after isolation but had no effect on cells assayed either two or

Table 2. Time Course of Change in H_2O_2 Production Postradiation

Time Postirradiation (h)	Radiation Dose (Gy)						n*
	2.5	5.0	7.5	10	20	50	
4	90 $\pm$ 7	97 $\pm$ 11	80 $\pm$ 5	68 $\pm$ 4	76 $\pm$ 4	66 $\pm$ 7	9
8	89 $\pm$ 9	92 $\pm$ 6	95 $\pm$ 10	82 $\pm$ 11	81 $\pm$ 11	67 $\pm$ 7	9
24	114 $\pm$ 6	145 $\pm$ 8	167 $\pm$ 9	152 $\pm$ 8	163 $\pm$ 12	153 $\pm$ 10	9
48	149 $\pm$ 5	179 $\pm$ 18	233 $\pm$ 30	330 $\pm$ 70	326 $\pm$ 48	358 $\pm$ 28	12
96	139 $\pm$ 5	163 $\pm$ 6	185 $\pm$ 5	250 $\pm$ 11	296 $\pm$ 13	373 $\pm$ 20	12
144	181 $\pm$ 13	262 $\pm$ 31	239 $\pm$ 33	463 $\pm$ 62	810 $\pm$ 94	1183 $\pm$ 290	12

Data are expressed as a percentage of control (unirradiated) values. Cells were irradiated 24 hours after isolation and assayed for PMA-induced (100 ng/mL for 60 minutes) H_2O_2 production at indicated times.

*Number of data points for each time point.

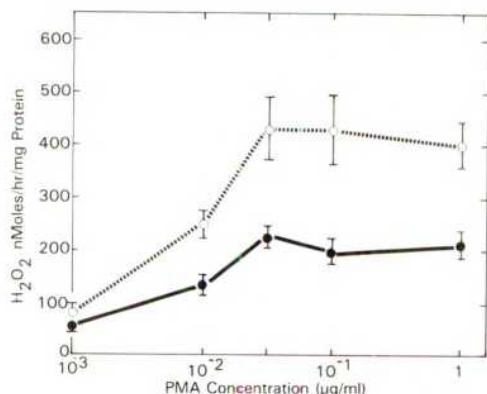


Fig 4. PMA dose-response curve of H_2O_2 release for control (●) and irradiated (○) cells. Cells were irradiated one day after isolation and assayed four days postirradiation. Each point represents the average of three different experiments $\pm$ SE.

five days after isolation (corresponding to day 1 and day 4 postirradiation).

Catalase, glutathione peroxidase, and SOD measurements. Three enzymes that play an important role in H_2O_2 metabolism are SOD, glutathione peroxidase, and catalase.¹³ To determine whether in H_2O_2 release in irradiated macrophages was due to either an increase in SOD activity or a decrease in glutathione peroxidase or catalase activity, the activity of these enzymes in cell homogenates was measured. As shown in Table 3, the activities of all three of these enzymes were identical in control and irradiated (20 Gy) cells.

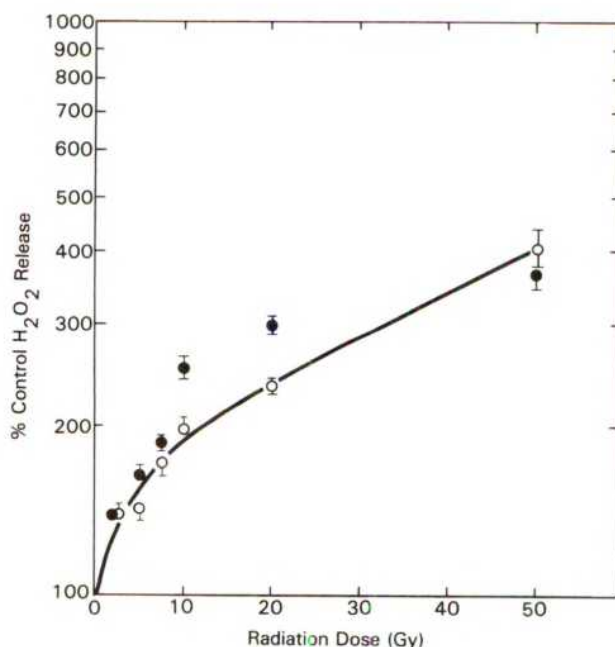


Fig 5. Effect of radiation on PMA-induced H_2O_2 release. (○) Data were obtained at four days postirradiation and expressed as a percentage of control. Points represent the average $\pm$ SE of three separate experiments. Cells were plated at different densities so that the protein per dish remained constant. (●) Data from the same experiments as in Fig 2.

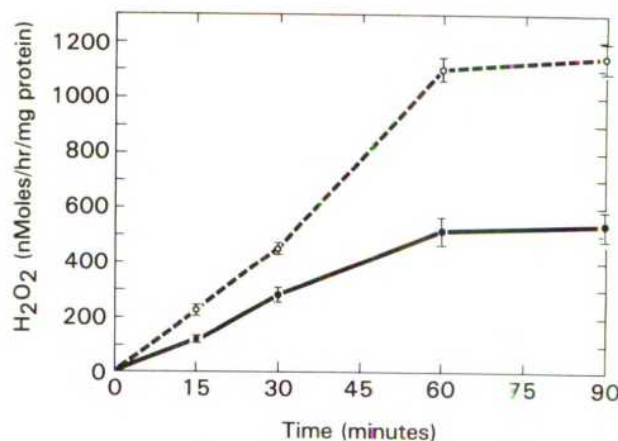


Fig 6. Time course of PMA-induced H_2O_2 release for control (solid line) and irradiated (20 Gy, dashed line) cells assayed at four days postirradiation. Cells were irradiated one day after isolation. Each point represents the mean $\pm$ SE of five measurements.

Superoxide measurements. The H_2O_2 released after PMA stimulation is produced by the dismutation of superoxide formed from the activation of an NADPH oxidase.¹⁴ If the increase in H_2O_2 levels postirradiation were due to an increase in the oxidase activity, the levels of superoxide released by irradiated cells might be increased as well. PMA-induced superoxide release was measured on cells four days postirradiation (20 Gy). In these studies, irradiated cells were plated at a higher density than were the control cells since Johnston et al¹⁰ demonstrated that superoxide release in mouse peritoneal macrophages varies inversely with cell density. As reported previously in human macrophages,⁹ the levels of superoxide released in response to PMA were lower (two- to fourfold) than the corresponding H_2O_2 levels in all groups of cells. The superoxide data showed more variability than the H_2O_2 data (possibly due in part to the lower levels). In five of eight studies, irradiated cells showed an increase in superoxide production over control cells (with increases ranging from 10% to 200%). The data shown in Table 4 summarize the data of the eight studies. The average percent changes of irradiated cells compared with control cells for superoxide, H_2O_2 , and protein per dish are given. It is evident that exposure to 20 Gy of γ -radiation did not produce a significant increase in superoxide release.

Table 3. SOD, Catalase, and Glutathione Peroxidase Activity in Radiated and Control Macrophages

	SOD*	Catalase†	Glutathione Peroxidase‡
Control	36.5 $\pm$ 11	1.02 $\pm$ 0.12	13.9 $\pm$ 0.6
Radiated	37.9 $\pm$ 11	1.23 $\pm$ 0.31	15.9 $\pm$ 1.1

Values are means $\pm$ SEM of three different experiments.

*Values are units per milligram protein. Cells were irradiated with 20 Gy one day after isolation and assayed four days later.

†Values are in nanomoles H_2O_2 degraded per minute per milligram protein. Cells were irradiated with 20 Gy five to six days after isolation and assayed four days later.

‡Values are in nanomoles NADPH consumed per minute per milligram cell protein. Cells were irradiated with 20 Gy one day after isolation and assayed four days later.

Table 4. Superoxide and H_2O_2 Production in Radiated Macrophages

	Control (%)	Protein per Dish, Control (%)*
O_2^-	137 $\pm$ 31	110 $\pm$ 6
H_2O_2	216 $\pm$ 33†	93 $\pm$ 7

Cells irradiated with 20 Gy 24 hours after isolation and assayed four days later. Data are means $\pm$ SE from eight different experiments.

*Dishes containing irradiated cells were plated at a higher cell density ($1.3 \times$ control).

† $P = .008$.

Effects of γ IFN. Experiments were carried out to determine whether the PMA-induced H_2O_2 release in irradiated cells could be further increased by pretreating the cells with γ IFN. The γ IFN studies were done by irradiating cells at varying times after isolation, culturing cells with γ IFN (100 U/mL) for one to four days, and measuring the PMA-induced H_2O_2 release from γ IFN-treated and control cells. Figure 7 shows the data from one study in which cells were cultured with γ IFN immediately after irradiation and then assayed four days later. γ IFN increased the PMA-induced H_2O_2 levels in all three groups of cells but had no effect on H_2O_2 levels in the absence of PMA. The combined data from this and four additional studies show that γ IFN increased PMA-induced H_2O_2 levels by 402%, 263%, and 199% in the control, 7.5-Gy, and 20-Gy groups, respectively. Thus, the irradiated cells responded to γ IFN. However, the levels of H_2O_2 produced by irradiated cells in the presence of γ IFN

were the same as for the nonirradiated cells exposed to γ IFN.

Effects of supernatant from irradiated cells. To determine whether radiated cells release a factor(s) into the supernatant that increases PMA-induced H_2O_2 release by macrophages, cell-free supernatants from irradiated cells were harvested on days 1 and 3 postirradiation and added to unirradiated cells. The average of six different studies indicated that PMA-induced H_2O_2 release in control cells refed irradiated supernatant was no different ($111\% \pm 18\%$) from control cells refed fresh media on days 1 and 3.

DISCUSSION

It is generally thought that macrophages are relatively radioresistant cells.¹⁵ However, recent studies indicate that time-dependent changes in bacterial killing,⁴ phagocytosis,¹ production of colony-stimulating factor,¹⁶ and Ia expression² occur after in vitro exposure to radiation. In most of these studies it was not possible to determine whether radiation was directly affecting the macrophage. In this paper, the effects of γ -irradiation on Percoll- and adherence-purified human peripheral blood monocytes ($\sim 95\%$ esterase-positive) were assessed so as to minimize the possibility that radiation is affecting a different cell type that is then influencing the macrophage.

Doses of γ -radiation as low as 2.5 Gy produced significant decreases in the number of adherent cells and the protein per dish by 48 hours postirradiation. The D_0 for the number of adherent cells remaining on days 4 and 6 postirradiation was 29 Gy. These results confirm previous studies¹⁵ demonstrating that, in terms of the number of viable cells, macrophages are relatively radioresistant. Our data differ from those of Kwan and Norman¹⁷ who calculated two D_0 values, 0.55 and 6.5 Gy for human monocytes four days postirradiation. However, in their studies, a mixed monocyte-lymphocyte fraction was irradiated, the cells were cultured in plastic tubes for four days, and then the number of large cells (or cells ingesting latex beads) was taken as a measure of the number of macrophages. The discrepancy between our findings and those of Kwan and Norman¹⁷ may be due to some of these differences.

In contrast to the effects of radiation on cell number, these studies demonstrate that doses of γ -radiation as low as 2.5 Gy produce a significant increase in PMA-induced H_2O_2 release. This increase was not due to a shift in the PMA dose response because (as shown in Fig 4) both control and irradiated cells had qualitatively similar dose-response curves. No detectable H_2O_2 was released from either irradiated or control cells in the absence of PMA, which indicated that radiation had no effect on basal H_2O_2 levels. It has been shown previously that there is an inverse relationship between the level of superoxide, another reaction oxygen intermediate, and the cell density of cultured macrophages.¹⁰ Therefore, experiments were done to (a) determine whether cell density affects H_2O_2 production and (b) examine the effects of radiation in the absence of any change in cell protein per dish. It should be noted that in the studies where the cell protein per dish was constant, the cell density in the irradiated cells will increase slightly since the protein per cell

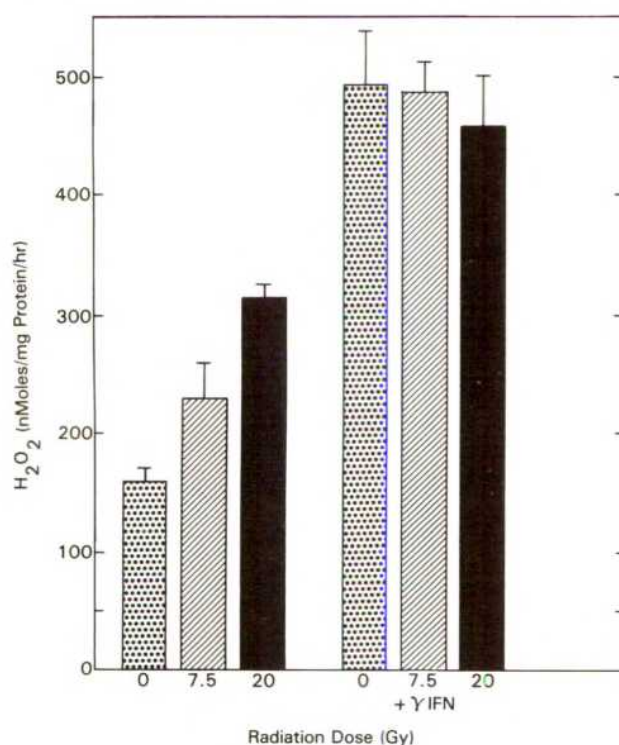


Fig 7. Effects of γ IFN PMA-induced H_2O_2 release by control and irradiated macrophages. Cells were irradiated one day after isolation. (A) Cells were incubated in 100 U/mL of γ IFN from days 0 to 4 postirradiation and assayed on day 4. Values represent the mean $\pm$ SE of four measurements.

decreased by 18% to 37% depending on the radiation dose. These studies demonstrated that, although PMA-induced H_2O_2 release was affected somewhat by cell density (when the protein per dish was less than 15 μ g), radiation still produced significant increases in H_2O_2 release when the protein per dish was unchanged (Fig 6). Comparison of Figs 2 and 5 shows not only that PMA-induced H_2O_2 release is more sensitive to radiation than is cell number but also that the relationship between PMA-induced H_2O_2 release and γ -irradiation is more complex than the relationship between cell number and γ -irradiation. The curve in Fig 6, obtained from data with comparable protein values, fits a two-exponent function, thereby indicating that radiation has multiple effects on the cells or that more than one population of cells (in terms of the H_2O_2 response) exists.

To investigate the possibility that factors released from the irradiated macrophages (or possibly a contaminating cell type) were responsible for the increase in PMA-induced H_2O_2 release postirradiation, experiments were done in which control cells were exposed to supernatant from irradiated cells. These studies indicated that the effects of radiation were not due to the release of a stimulatory factor into the media since supernatants from irradiated cells had no effect on control cells. In addition, this finding supports the view that radiation is probably acting directly on the macrophage and not on a contaminating cell type.

To determine the site in the oxidative pathway responsible for the increase in the H_2O_2 -releasing capacity of irradiated macrophages, studies were done to assess the release of superoxide by PMA-stimulated cells after radiation and to measure SOD, glutathione peroxidase, and catalase levels in these cells. Those experiments indicated that at four days postirradiation (20 Gy) the SOD, glutathione peroxidase, and catalase levels as well as the PMA-induced superoxide release were not significantly different from control values. Further studies will need to be done that measure the NADPH oxidative activity in radiated and control cells to elucidate the site(s) of action of radiation on the oxidative pathway.

Interestingly, γ IFN, which enhances PMA-induced H_2O_2 release in macrophages,^{6,18} also increases H_2O_2 levels in irradiated (20 Gy), cells. Thus, irradiated cells are still responsive to γ IFN. However, γ IFN has a larger effect on

the control cells so that the levels of H_2O_2 released by both control and irradiated cells in the presence of γ IFN are identical. A possible interpretation of these results is that there is a maximum amount of H_2O_2 that can be released by the macrophage. Therefore, irradiated cells that release more H_2O_2 in the absence of γ IFN produce less H_2O_2 in response to γ IFN.

The production of reactive oxygen intermediates by phagocytes is associated with increased activation since it has been linked to tumor cell cytotoxicity, the killing of bacteria, and intracellular pathogens.^{5,6} However, chronic stimulation of macrophages can lead to tissue damage and inflammation. It is possible that the stimulatory action of radiation on the oxidative burst of macrophages is partly responsible for the tissue damage that occurs postirradiation. For example, the mutagenic effects of ionizing radiation are due in great part to the direct formation of active oxygen intermediates,¹⁹ and mutagenicity may be further enhanced by additional free radicals produced by phagocytic cells. Recent studies have shown that reactive oxygen intermediates produced by stimulating neutrophils with phorbol esters are mutagenic (transforming mouse fibroblasts into malignant cells).²⁰

Several studies have demonstrated an increased activation in macrophages harvested from irradiated animals. Therefore, the in vitro increases in H_2O_2 production reported in this study may have in vivo correlates. For example, Schultz et al²¹ have shown that doses of x-irradiation of 1 to 8 Gy increase the ability of macrophages to suppress the proliferation of MBL-2 target cells. In these studies, 6 Gy increased the cytostatic ability of macrophages from three hours to seven days postirradiation. Similar doses of whole body radiation also increased the lysosomal enzyme content of macrophages.²² In in vivo studies, the effects of damaged lymphocytes, infection, etc, on macrophage function cannot be separated from the direct effect of radiation on the macrophage, which makes it difficult to compare in vivo studies with the in vitro radiation data presented in this paper. However, it is evident that, if an increase in H_2O_2 production occurs postirradiation, it is likely to enhance the deleterious effects of radiation.

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The Transglutaminase in Vascular Cells and Tissues Could Provide an Alternate Pathway for Fibrin Stabilization

By Charles S. Greenberg, Komandoor E. Achyuthan, Michael J. Borowitz, and Marc A. Shuman

A thrombin-independent transglutaminase (TG) has been identified in vascular cells and tissues from human, rabbit, rat, porcine, and bovine sources. The vascular TG had several properties that were similar but not identical to guinea pig liver TG. Both enzymes had similar chromatographic and electrophoretic properties, preferentially cross-linked the α -chains of fibrinogen, and reacted with polyclonal and monoclonal anti-guinea-pig liver TG antibodies. However, the TG from adult bovine aortic endothelial (ABAE) cells exhibited a novel $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependence for enzymatic activity that was distinct from that of purified guinea pig liver TG. The mol wt of the vascular TG (79 ± 3 kd) determined by sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE) was slightly lower than the purified guinea pig liver TG (85 ± 9 kd). The TG antigen was detected by immunohistochemical techniques in association with the endothelial and smooth muscle cells of arteries, veins, venules, and capillaries. The TG antigen also codistributed with the fibronectin antigen along the hepatic sinusoids. The ABAE cell TG cross-linked α_2 -plasmin inhibitor to fibrinogen and caused the modified fibrinogen to be 40-fold more resistant to plasminolysis. A thrombin-independent TG in vascular cells of blood vessels could provide an alternate pathway to inhibit fibrinolysis and promote fibrin stabilization.

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TRANSGLUTAMINASES (TGs) (R-glutaminyl-peptide: amine- γ -glutamyltransferase, E.C. 2.3.2.13) catalyze the formation of covalent bonds between peptide-bound glutamyl residue and either a lysyl group in a protein or a primary amino group in polyamines. Although TG activity has been detected in several tissues and cells,^{1,2} the physiologic function of the cell-associated TG is poorly defined.¹ The plasma TG, factor XIIIa, has been traditionally regarded as the enzyme that catalyzes the final steps in the formation of a stable fibrin clot. The important factor XIIIa-mediated reactions that stabilize fibrin include cross-linking of fibrin monomers to each other³ as well as cross-linking of fibronectin⁴ and α_2 -plasmin inhibitor⁵ to fibrin.

Laki et al⁶ first reported that TG activity was increased in the sclerotic portion of aortas from cholesterol-fed rabbits as compared with the healthy-appearing segments. However, the biochemical and immunologic characterization of TG activity was not established. Shainoff and Page⁷ reported that insoluble fibrinogen was present in atherosclerotic

human aortas and proposed that a thrombin-independent TG could have led to fibrinogen accumulation in the wall of the blood vessel. These observations prompted us to pursue the characterization of the TG activity in vascular cells and tissues.

We have found that cultured adult bovine aortic endothelial (ABAE) cells and vascular smooth muscle (VSM) cells contain a thrombin-independent TG that is biochemically and immunologically similar but not identical to the guinea pig liver TG. The TG antigen was localized to endothelial and smooth muscle cells in human arterial and venous tissues and along the sinusoids in the liver. Vascular TG cross-linked fibrinogen to itself and also cross-linked α_2 -plasmin inhibitor to fibrinogen. The α_2 -plasmin inhibitor cross-linked fibrinogen was 40-fold more resistant to plasminolysis than was native fibrinogen. The vascular TG could provide an alternate pathway toward fibrin stabilization and inhibit fibrinolysis.

MATERIALS AND METHODS

Tissue culture dishes were purchased from Falcon Plastics, Oxnard, CA. Tissue culture medium and trypsin were obtained from GIBCO (Grand Island, NY), and calf serum and fetal calf serum (FCS) were from Hyclone Laboratories (Logan, UT). [³H]-Putrescine, Aquasol 2, and Protosil were from New England Nuclear (Boston). Hammerstein casein was purchased from United States Biological Laboratories (Cleveland). BioRad protein assay kit, nitrocellulose membranes, and Enzymobead reagent were from BioRad, Richmond, CA. Diethylaminoethyl (DEAE)-Sephacel was obtained from Sigma, St Louis. Human α -thrombin was supplied by Dr John Fenton, II, New York State Department of Health, Albany, NY. Sephadex G-25M, PD-10 columns were from Pharmacia Fine Chemicals, Piscataway, NJ. Factor XIII-free fibrinogen was prepared as previously described.⁸ Rabbit aortic and human aortic and venous tissues were generously provided by Dr Per Otto Hagen, Department of Surgery, Duke University. Pig aorta was purchased from the local slaughterhouse. Affinity-purified anti-guinea-pig liver TG antibody⁹ was a generous gift from Dr Peter Davies, Department Pharmacology, University of Texas, Houston. Monoclonal antibody (CUB-7401) against guinea pig liver TG¹⁰ was kindly supplied by Dr Paul Birckbichler, Samuel Roberts Noble Foundation, Ardmore, OK. Goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase conjugates were from BioRad. Anti-factor XIII (A-subunit) antisera from rabbits, rabbit antifibrinogen antisera, and rabbit anti-human fibronectin antisera

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were purchased from Calbiochem, La Jolla, CA. Purified human α_2 -plasmin inhibitor was purchased from American Diagnostica, Greenwich, CT. Rabbit antisera to human α_2 -plasmin inhibitor was obtained from American Diagnostica, Greenwich, CT.

Tissue culture techniques. ABAE cells and bovine vascular smooth muscle (VSM) cells were cultured by previously described methods on 10-cm plastic culture plates.^{11,12} ABAE and VSM cells were used between passages 2 and 15. Confluent ABAE cells reached a density of 900 cells/mm². ABAE cells were characterized by the presence of Weibel-Palade bodies¹³ and factor VIII-related antigen.¹⁴ VSM cells were characterized by their typical morphology and absence of factor VIII-related antigen. ABAE and VSM cells were cultured with fibroblast growth factor purified from bovine brain.¹⁵ Porcine aortic endothelial cells were cultured by previously described methods in tissue culture flasks and used during the third passage.¹⁴ Rabbit vascular (thoracic aorta) smooth muscle cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS.

Cell harvesting. Cell monolayers were dissociated by exposure (2 to 3 minutes at 22°C) to trypsin. When cells were rounded (monitored by inverted-phase contrast microscope), the trypsin solution was removed and 5 mL medium supplemented with 10% serum was added to inhibit trypsin. Cells were washed three times with 20 mmol/L Tris-HCl, 130 mmol/L NaCl, pH 7.4 (TBS). After the third wash, the cells from each dish were resuspended in 0.1 to 0.2 mL of 50 mmol/L Tris-HCl, 25% glycerol, pH 8.5. Phenylmethyl sulfonyl fluoride (PMSF) (0.2 mmol/L) was added immediately prior to sonication to limit proteolysis. In a few experiments, cells were removed from tissue culture dishes by scraping with either a rubber policeman or a cell scraper after the monolayers were washed twice with media.

TG assay. Washed and resuspended cells were sonicated three times at 4°C for 15 seconds at 40% output using a model W-220 sonicator, Heat Systems-Ultrasonics, Farmingdale, NY. The protein concentration of the sonicated cells was determined using the BioRad Protein Assay. Cell sonicates (10 to 100 μ g) were assayed for TG activity in presence of either 5 mmol/L CaCl₂ or 10 mmol/L EDTA, as described previously.¹⁶

Agarose gel electrophoresis. Sonicates of cultured ABAE cells were incubated with DEAE-Sephacel in TBS and washed. The protein bound to the DEAE was eluted with 0.4 mol/L NaCl. The eluate was subjected to electrophoresis in gels cast from *N,N*'-dimethylcasein (0.3%) and agarose (1%) in 75 mmol/L barbital buffer containing 1 mmol/L EDTA.¹⁷ After electrophoresis, the gels were stained for TG activity using monodansylcadaverine.¹⁷

Immunoblotting of ABAE cell TG, guinea pig liver TG, factor XIII, fibrinogen and α_2 -plasmin inhibitor antigens after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ABAE cell sonicates or guinea pig liver TG were taken up in a solution containing 1% sodium dodecylsulfate (SDS), 1.5 mol/L urea, 2.5 mmol/L EDTA, 30 mmol/L Tris, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, and were boiled for 5 minutes. The material was then fractionated by slab-gel electrophoresis in an 8% discontinuous polyacrylamide gel (SDS-PAGE) using a 3% stacking gel.¹⁸ The proteins were then transferred to nitrocellulose membrane.¹⁹ The membrane was then incubated with bovine serum albumin (BSA) (3%) in TBS and later reacted with a monoclonal antibody to guinea pig liver TG (CUB-7401) or control unreactive antibody (CUB-11) at 1:1,000 dilution for 8 to 12 hours at 4°C with gentle shaking. The membrane was washed with TBS and then reacted with goat anti-mouse horseradish peroxidase conjugate (1:2,000 dilution) 6 hours at 4°C. In other experiments, the membrane was reacted with affinity-purified anti-guinea-pig liver TG antibody followed by reaction with rabbit anti-goat horseradish peroxidase conjugate, in the manner described above. A similar

procedure was used to detect factor XIII, α_2 -plasmin inhibitor and fibrinogen antigens. The membrane was reacted with either anti-factor XIII antisera (1:2,000 dilution) anti- α_2 -plasmin inhibitor antisera (1:1,000 dilution), or antifibrinogen antisera (1:1,000), followed by reaction with goat anti-rabbit horseradish peroxidase conjugate (1:2,000 dilution). After being washed, the membranes were stained with a color reagent from BioRad.

Purification of TG. Plasma factor XIII was purified as previously described⁸ and stored at -70°C. Guinea pig liver TG was purified by DEAE column chromatography.²⁰

HPLC gel filtration and DEAE column chromatography of guinea pig liver TG and ABAE TG. Confluent ABAE sonicates and purified guinea pig liver TG preparations were centrifuged at 150,000 g for 60 minutes in a Beckman air-driven ultracentrifuge. The supernatant containing at least 90% of the total enzyme activity was injected into an HPLC gel filtration column (BioSil TSK-125, BioRad) and eluted with 50 mmol/L Tris-HCl pH 8.5. Five-hundred-microliter fractions were collected at a flow rate of 1.0 mL/min. The fractions were then assayed for TG activity.

Confluent ABAE cell TG was partially purified by adsorption to a column of DEAE-Sephacyl. The sonicate was applied to a 15-mL column of DEAE-Sephacyl previously equilibrated with 50 mmol/L Tris-HCl, 1 mmol/L EDTA, 200 μ mol/L PMSF, pH 8.5. The column was then washed with 200 mL of the same buffer, and the enzyme was eluted using a linear (0 to 1 mol/L) sodium chloride gradient in the same buffer. Two-milliliter fractions were collected at a flow rate of 30 mL/hour. The column was operated at 4°C. The fractions were then checked for TG activity. Purified guinea pig liver TG was also applied to the same column under similar conditions and its elution was monitored similarly.

Identification of fibrinogen cross-linking pattern. ABAE cell sonicate (12.5 μ g) was incubated with purified fibrinogen (60 μ g) in the presence of 25 mmol/L DTT, 1.0 mmol/L calcium chloride at 22°C for 60 minutes. The reaction was stopped as described in the preceding section. SDS-PAGE was performed in an 8% polyacrylamide gel. The gel was stained with Coomassie blue and then destained. Purified fibrinogen was electrophoresed under similar conditions and served as markers for α -, β -, and γ -chains. High- and low-mol-wt protein mixtures (BioRad) were electrophoresed simultaneously.

Identification of TG activity in vascular tissues. Human saphenous vein (672 mg) was cut into 2-mm pieces and homogenized five times in 3.0 mL of 0.1 mol/L Tris-HCl, pH 8.5, using a Tissumizer (Tekmar, Cincinnati) at 80% output for 30 seconds. The homogenate was sonicated three times for 15 seconds at 60% output using a cell sonicator. The sonicated material was centrifuged for 15 minutes at 10,000 g, and the supernatant was filtered through a Millex-HA 0.45 μ mol/L filtration unit. All procedures were performed at 4°C. The final extract, which had a protein concentration of 3.72 mg/mL, was kept at 4°C until assayed. The enzyme was assayed within 2 hours of homogenization. All other tissues were homogenized and sonicated as described above.

Immunohistochemistry of TG and fibronectin antigens in human tissues. Immunoperoxidase testing was performed by the standard "ABC" method as previously described²¹ using frozen sections of various human and animal tissues. When liver was used, endogenous biotin binding was first blocked using excess avidin as described.²¹ Sections were stained with diaminobenzidine and 0.03% H₂O₂ and counterstained with hematoxylin. As controls, isotype-specific monoclonal antibodies or culture supernate from the nonreactive fusion partner were used in place of the monoclonal anti-TG antibody supernate.

The distribution of TG in human liver was compared with that of fibronectin. In these experiments, frozen sections were cut as above and, after preincubation with normal goat serum, were stained with

rabbit antifibrinogen antisera at a dilution of 1:2000, followed by biotinylated goat anti-rabbit IgG, and then avidin and biotinylated horseradish peroxidase. Normal rabbit serum and/or mouse monoclonal antibodies were also substituted as controls. Tissues for these studies came from the frozen bank in the immunopathology laboratory at Duke University Medical Center.

Enzyme-linked immunosorbent assay for tissue TG. Enzyme-linked immunosorbent assay (ELISA) for guinea pig liver TG was conducted as described by Birckbichler et al.¹⁰

Effect of TG-catalyzed cross-linking of α_2 -plasmin inhibitor to fibrinogen on plasminolysis. Fibrinogen (32 μ g) was incubated with confluent ABAE cell sonicate (5 μ g cell protein) and α_2 -plasmin inhibitor (4 μ g) in a total volume of 50 μ L of 0.02 mol/L Tris-HCl, pH 7.4, containing 130 mmol/L NaCl and 5 mmol/L calcium chloride, for 60 minutes at 37 °C. In some incubations, 10 mmol/L dithiothreitol was also present. Reactions were also performed in the presence of 5 mmol/L EDTA. The reactions were stopped by 1% SDS, 1.5 mol/L urea, 2.5 mmol/L EDTA, 30 mmol/L Tris, 5% 2-mercaptoethanol, and 0.001% bromophenol blue, and boiled for 5 minutes. Samples were separated by SDS-PAGE on a 6% to 15% linear polyacrylamide gradient and subjected to immunoblotting as described previously.

ABAE cell sonicate (10 μ g) was incubated with fibrinogen (100 μ g) and purified α_2 -plasmin inhibitor (10 μ g) in a total volume of 50 μ L of 0.02 mol/L Tris-HCl, 0.13 mol/L NaCl pH 7.4, 5 mmol/L CaCl_2 for 30 minutes at 37 °C. Similar reactions were performed either in the presence of 5 mmol/L EDTA or by omitting one of the components from the assay. Fibrinogen was precipitated by protamine sulfate (2 mg/mL), and washed by centrifugation in 0.1 mol/L Tris-HCl, pH 7.4, containing protamine sulfate 2 mg/mL. Fibrinogen was resuspended in 0.02 mol/L Tris-HCl, 0.13 mol/L NaCl, pH 7.4, at a concentration of 0.4 mg/mL and incubated with plasmin (0.005 to 0.16 CU/mL) for 10 minutes at 37°C. Samples were solubilized in 1% SDS, 1.5 mol/L urea, 2.5 mmol/L EDTA 30 Tris, pH 6.8, and electrophoresed on a 4% to 15% gradient SDS-polyacrylamide gel. Gel was stained with Coomassie blue and dried, and the degradation of the fibrinogen was quantitated by scanning densitometry. The absorbance of the fibrinogen bands in the presence of plasmin was divided by the absorbance of fibrinogen in the absence of plasmin and multiplied by 100 to yield the percentage degraded.

RESULTS

Enzymatic properties of vascular TG. A thrombin-independent, calcium- and thiol-dependent TG activity was detected in sonicates of ABAE and VSM cells by incorporation of [³H]-putrescine into dimethylcasein. The ABAE and VSM TG activity was maximal between pH 8.5 and 9.0. The TG assay was sensitive and detected 40 ng of either purified guinea pig TG, human factor XIIIa, or the TG activity in 1 to 10 μ g of ABAE or VSM cell protein. Incubating sonicates of ABAE and VSM cells with 1 mmol/L *N*-ethylmaleimide or 1 mmol/L iodoacetic acid inhibited 98% of the TG activity. TG activity was not detected in the culture media before or after ABAE cells were cultured for 10 days.

Immunoblotting using polyclonal anti-factor XIII a-chain antisera did not detect the factor XIII antigen in 200 μ g of confluent ABAE cell protein. This immunoblotting technique was capable of detecting 50 ng of purified human or bovine factor XIII. Thrombin, which is necessary for the activation of plasma and platelet factor XIII, did not modify the TG activity of ABAE cell sonicates. In contrast to factor

XIIIa, which initially catalyzes γ -chain cross-linking of fibrinogen, the vascular TG selectively cross-linked the α -chains of fibrinogen and not the β - or γ -chains (Fig 1).

More than 90% of the TG activity remained in the cytoplasmic fraction of confluent ABAE cells after centrifugation for 60 minutes at 150,000 g. The presence of calcium chloride (5 mmol/L) or EDTA (5 mmol/L) had no effect on the distribution of the TG from disrupted ABAE cells after centrifugation. To determine whether there was latent TG activity in the particulate or soluble fraction of ABAE cell sonicates, each fraction was incubated with 0.5% NP-40 or 0.5% Triton X-100 (Sigma, St Louis) to solubilize the TG. The nonionic detergent treatment did not change the TG activity in either fraction.

Presence of TG in vascular tissues and cells. TG activity was also detected in homogenates from both human and several other mammalian blood vessels (Table 1). Thrombin was not required for the expression of the TG activity from any of the cells or tissues. The TG activity was only detected in fresh sonicates of tissues and cells and was destroyed by freezing at -70 °C unless glycerol (25% vol/vol) was present during freezing. TG activity was also detected in a variety of other cultured vascular cells, including human umbilical vein endothelium (Table 1). Aortic endothelial and smooth muscle cells could be the major source of TG activity in bovine aortic tissues since they had a higher specific activity than the original tissue. The specific activity of confluent ABAE cells remained constant for up to 15 cell passages. However, the specific activity of nonconfluent cells was sevenfold lower (data not shown). Results for all the cells described in Table 1 were from postconfluent cells.

Immunologic characterization of vascular TG. Immunologic similarity between ABAE cell TG and guinea pig liver TG was demonstrated by immunoblotting techniques

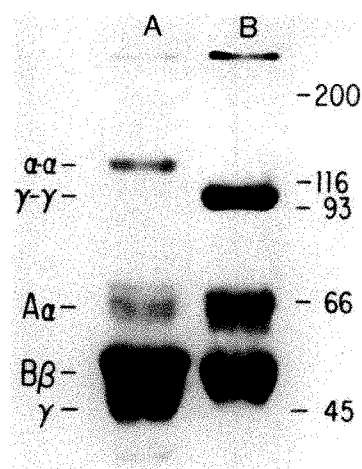


Fig 1. Pattern of fibrinogen cross-linking by confluent ABAE cell sonicate (lane A) and purified platelet factor XIIIa (lane B). The mobility of the mol wt standards are shown $\times 10^{-3}$ on the right margin. Purified fibrinogen was incubated with either ABAE sonicate or purified platelet factor XIIIa at 22°C for 1 hour. The incubation mixture was separated by SDS-PAGE as described in the text. Location of the $A\alpha$ -, $B\beta$ -, and γ -chains of fibrinogen as well as the $\gamma\gamma$ - and $\alpha\alpha$ dimers are shown on the left margin.

Table 1. Distribution of TG Activity in Cells and Tissues

Tissue/Cell	Specific Activity*
Tissues	
Human abdominal aorta	0.80
Porcine abdominal aorta	2.64
Rabbit abdominal aorta	2.15
Rabbit inferior vena cava	4.10
Bovine abdominal aorta	1.96
Cells	
Porcine aortic endothelial cells	2.24
Porcine VSM	1.82
Rabbit VSM cells	1.30
Adult ABAE	122.00
Bovine VSM	36.40

Tissues and cells were homogenized, sonicated and assayed for enzyme activity as described in the Materials and Methods section.

*Specific activity is defined as nanomoles of putrescine incorporated into casein by 1 mg of cell protein in 1 hour at 37°C.

using the culture supernatant from a hybridoma cell line producing monoclonal anti-guinea-pig liver transglutaminase antibody (CUB-7401).¹⁰ The confluent ABAE cell extract revealed a major 79 ± 3 -kd band that migrated slightly ahead of the guinea pig liver TG (lane 1; Fig 2). Purified guinea pig liver TG migrated as a single 85 ± 9 -kd protein under these conditions (lane 2, Fig 2). Tissue culture media before culturing ABAE cells did not have any detectable TG antigen. However, the culture supernatant did have a very small amount of TG antigen that migrated as a higher mol wt antigen ~ 150 kd (data not shown). The TG antigen in rabbit VSM, porcine aortic endothelial cells, and an extract

of porcine aortic tissue also migrated as an 80-kd antigen. Occasionally, lower mol wt TG antigens were detected in the sonicates of ABAE cells. Inclusion of protease inhibitors in the cell lysis buffer did not completely reduce the appearance of these lower mol wt antigens. The confluent ABAE sonicates did not react with the culture supernatant from a nonreactive hybridoma cell line (CUB-11). The monoclonal antibody (CUB-7401) did not cross-react with 5 μ g of either factor XIII, fibrinogen, or fibronectin. The ELISA assay detected 0.3 ng TG antigen in 100 ng cell protein.

Chromatographic and electrophoretic characterization of vascular TG. High-pressure liquid chromatography (HPLC) gel filtration, DEAE column chromatography, and casein-agarose electrophoresis were used to ascertain whether there was a single functional form of TG in ABAE cells. TG activity in confluent ABAE cells eluted as a single peak from the HPLC gel filtration column with the same retention time (RT = 7 minutes) as purified guinea pig liver TG. More than 90% of the ABAE TG activity adsorbed to and eluted from a DEAE-Sepharose column as a single peak at a NaCl concentration (0.35 to 0.38 mol/L), similar to purified guinea pig liver TG.

Identification of TG activity after electrophoretic separation in nondenaturing casein-agarose gels has been used by others to characterize the TG from various tissues.¹⁷ ABAE cell TG partially purified by DEAE-Sepharose chromatography and purified guinea pig liver TG migrated in casein-agarose gels as a single band with an R_f of 0.6 relative to bromphenol blue dye. In contrast, purified factor XIIIa migrated more slowly, with an R_f of 0.1.

Effect of divalent cations on TG activity. We found a unique effect of calcium and magnesium ions on the TG in ABAE cells (Fig 3). A very narrow range of calcium ion

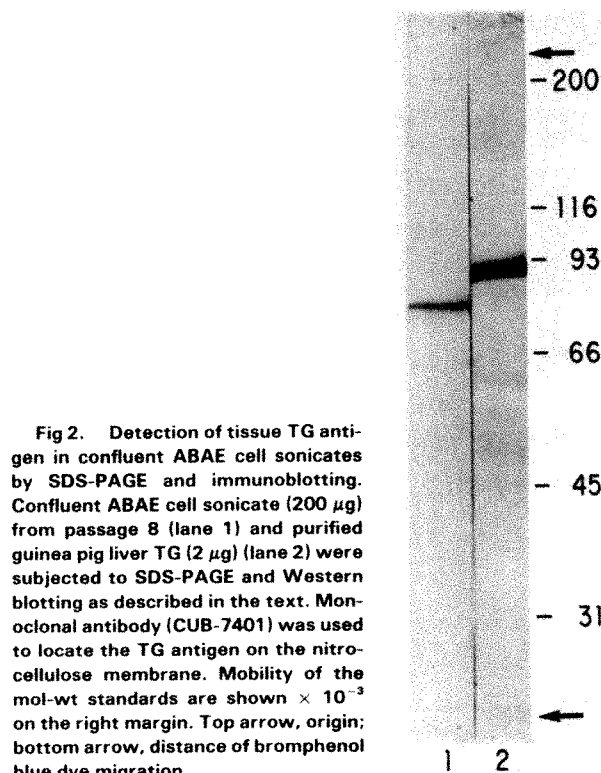


Fig 2. Detection of tissue TG antigen in confluent ABAE cell sonicates by SDS-PAGE and immunoblotting. Confluent ABAE cell sonicate (200 μ g) from passage 8 (lane 1) and purified guinea pig liver TG (2 μ g) (lane 2) were subjected to SDS-PAGE and Western blotting as described in the text. Monoclonal antibody (CUB-7401) was used to locate the TG antigen on the nitrocellulose membrane. Mobility of the mol-wt standards are shown $\times 10^{-3}$ on the right margin. Top arrow, origin; bottom arrow, distance of bromphenol blue dye migration.

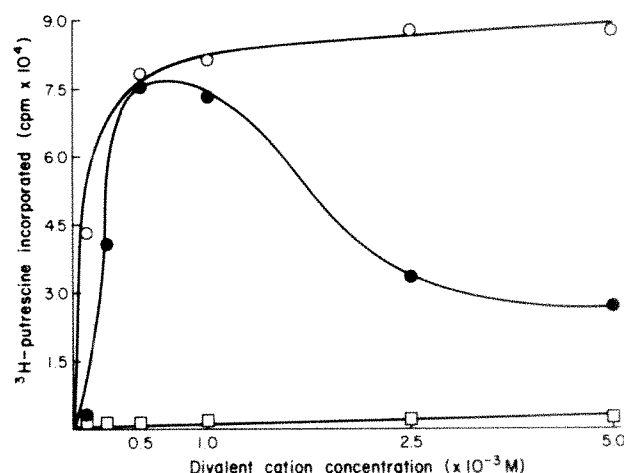


Fig 3. Effect of CaCl_2 and MgCl_2 concentration on ABAE cell TG activity. Fifty micrograms of protein from sonicates of ABAE cells was incubated with increasing concentrations of CaCl_2 (●—●), MgCl_2 (□—□) or MgCl_2 in presence of 0.1 mmol/L CaCl_2 (○—○). The amount of [^3H]-putrescine incorporated into dimethylcasein was determined after 30 minutes of incubation at 37°C. EDTA (1 mmol/L) was added to each reaction mixture to chelate unbound divalent cations. The CaCl_2 and MgCl_2 concentrations added to the reaction mixtures were 1.0 mmol/L higher than the desired final concentration.

concentration was required for optimum TG activity; and when this range was exceeded, a reduction in TG activity was observed. When magnesium ions were substituted for calcium ions, TG activity was not detected. However, in the presence of suboptimal concentrations of CaCl_2 (0.1 mmol/L), MgCl_2 promoted ABAE cell TG activity (Fig 3). This was consistently observed in five experiments, using both confluent ABAE and VSM cells. The Mg^{2+} -dependent increase in TG activity was also studied using partially purified ABAE cell TG obtained from ABAE cells following HPLC gel filtration. Magnesium chloride (1 mmol/L) and 0.5 mmol/L CaCl_2 increased TG activity to 64% of the level achieved by 1 mmol/L calcium chloride. Such an effect was not observed with purified guinea pig liver TG.

Identification and localization of TG antigen in human tissues. When human skin was analyzed by immunohistochemistry, the TG antigen was confined to the blood vessels in the dermis. A section of two blood vessels from the skin showed intense staining along the endothelial portion of a medium-sized vein (Fig 4, arrow). In the vessel of a medium-sized artery, the TG antigen localized mainly to the smooth muscle cell layer (Fig 4). A medium-sized artery showed positive staining with anti-TG monoclonal antibody along the endothelial surface. Staining was also observed along the surface of smooth muscle cells when a cross-section of the vessel was studied (data not shown). Human and rabbit aorta also stained for TG antigens in the endothelium, VSM cells, and interstitial connective tissue of the intima and media (data not shown).

Although TG was purified from guinea pig liver >10 years ago, the location of this enzyme in the liver was never established. We have detected TG antigen lining the sinusoids of human liver (Fig 5) as well as rat and guinea pig livers (data not shown). There was no detectable staining of hepatocytes, and the nonreactive antibody (CUB-11) produced no immunoreactivity (Fig 5). When liver tissues were stained with antifibronectin antibody, a reaction occurred similar to the pattern observed with the TG, suggesting that

both proteins were distributed in the same tissue compartment (Fig 5).

Effect of ABAE cell lysates on plasmin degradation of fibrinogen. The ABAE cell TG cross-linked purified α_2 -plasmin inhibitor to fibrinogen (Fig 6A and B). The high-mol-wt complexes (Fig 6A) were not observed when α_2 -plasmin inhibitor or CaCl_2 was omitted from the reaction or when the reaction was conducted with 5 mmol/L EDTA. Fibrinogen- α_2 -plasmin inhibitor complexes with mol wt of 132, 195, 260, and >260 kd were identified using either antifibrinogen or anti- α_2 -plasmin inhibitor antibody. Because these complexes reacted with both antibodies and since the α -chain (67 kd) is the preferred cross-linking site in fibrinogen for the ABAE TG (Fig 1), we believe these complexes to be α_2 -plasmin inhibitor cross-linked to the α -chain monomers or α -chain polymers of fibrinogen. The complexes of mol wt ≥ 260 kd represent α_2 -plasmin inhibitor cross-linked to α -chain polymers. The exact stoichiometry of α_2 -plasmin inhibitor cross-linked to fibrinogen cannot be determined from these results.

ABAE cell sonicates also made fibrinogen resistant to plasmin degradation. In the absence of either TG, calcium ions, or α_2 -plasmin inhibitor, plasmin (0.005 CU/mL) degraded 50% of the fibrinogen (Fig 6B). After α_2 -plasmin inhibitor was cross-linked to fibrinogen by ABAE cell TG, the plasmin concentration to degrade 50% of the fibrinogen was increased 40-fold (0.20 CU/mL) (Fig 6B). The formation of the cross-linked complexes between fibrinogen and α_2 -plasmin inhibitor and the resistance of modified fibrinogen to plasmin degradation were unaffected by dithiothreitol in the cross-linking reaction (data not shown).

DISCUSSION

Although previous investigators have detected TG activity in homogenates of rabbit aorta,⁶ the TG was neither biochemically characterized nor immunohistochemically localized. Cultured ABAE and VSM cells contain a thrombin-independent TG with biochemical and immunologic properties similar but not identical to guinea pig liver TG. This protein was biochemically and immunologically distinct from factor XIIIa.²² Both the vascular and guinea pig liver TG enzymes preferred uncharged polyamines (pH optimum 8.5 to 9.0), required a free sulfhydryl group for activity, reacted with polyclonal and monoclonal antibodies specific for guinea pig liver TG, were predominantly cytoplasmic enzymes, selectively cross-linked the α -chains of fibrinogen, and eluted from DEAE columns under similar conditions. Both enzymes were also inhibited in a noncompetitive manner by GTP.^{23,24}

We observed a novel $\text{Ca}^{2+}/\text{Mg}^{2+}$ effect on TG activity in confluent ABAE cells. With suboptimal concentrations (0.1 mmol/L) of calcium ions, an Mg^{2+} -dependent increase in TG activity occurred. To our knowledge, this is the first observation of this type of $\text{Ca}^{2+}/\text{Mg}^{2+}$ effect on TG activity. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ effect was not observed with purified guinea pig liver TG, demonstrating that this was not an artifact of our assay system. Since the $\text{Ca}^{2+}/\text{Mg}^{2+}$ effect was less prominent when the partially purified ABAE TG was stud-

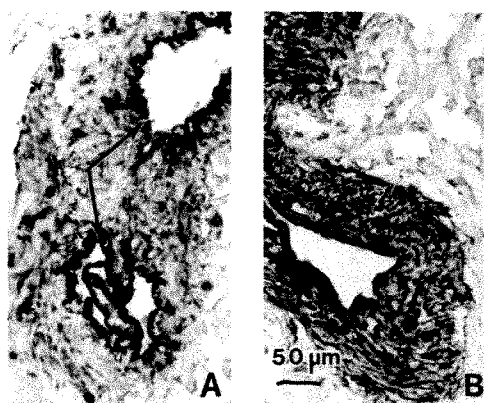


Fig 4. Frozen section of two blood vessels from human skin stained with anti-TG. (A) A medium-sized vein shows intense staining along the endothelial portion of the vessel (arrow) with less staining in the wall. In contrast, a medium-sized artery (B) shows localization mainly within the smooth muscle layer.

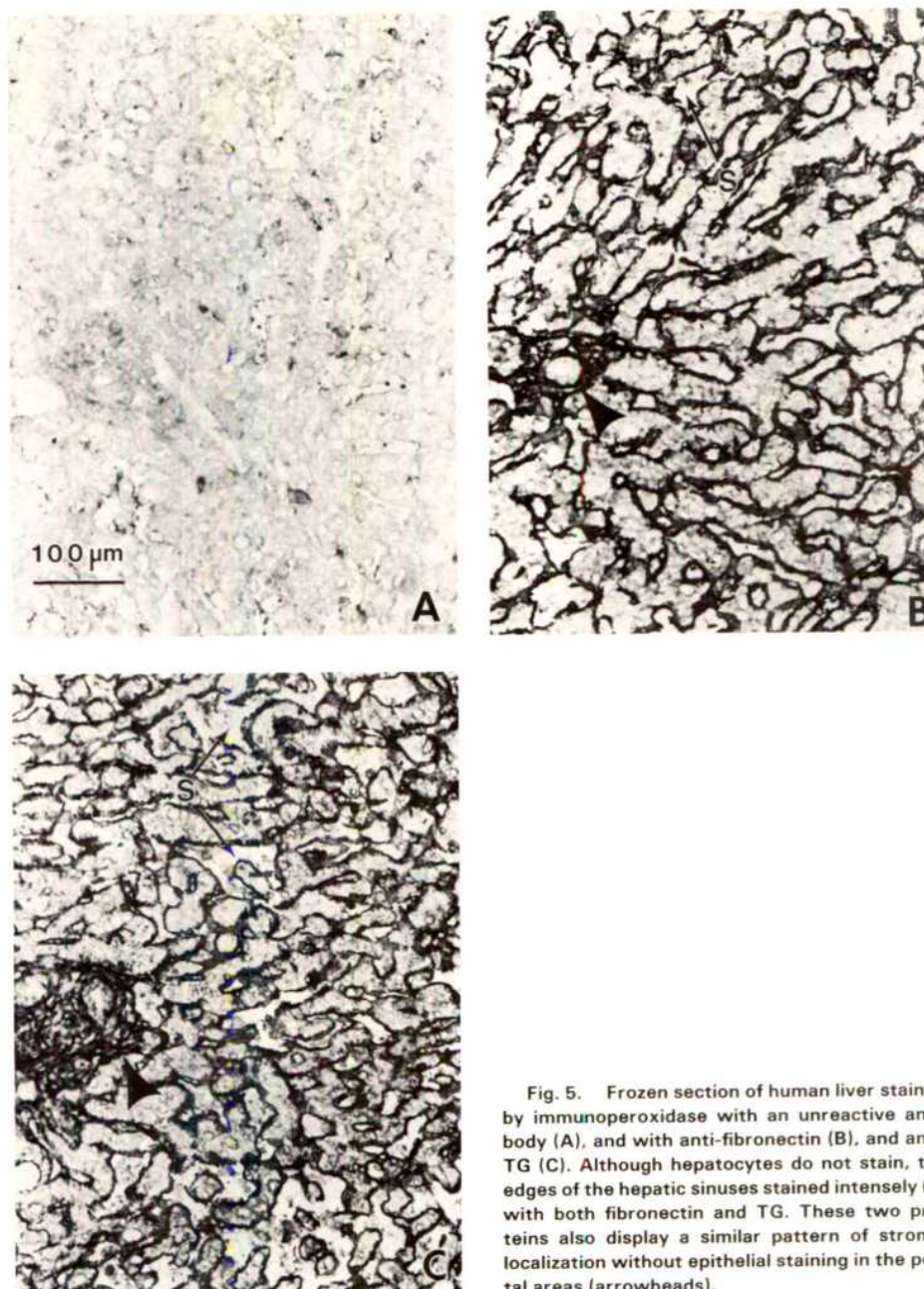


Fig. 5. Frozen section of human liver stained by immunoperoxidase with an unreactive antibody (A), and with anti-fibronectin (B), and anti-TG (C). Although hepatocytes do not stain, the edges of the hepatic sinuses stained intensely (S) with both fibronectin and TG. These two proteins also display a similar pattern of stromal localization without epithelial staining in the portal areas (arrowheads).

ied, Mg^{+2} ions may be modulating ABAE cell TG activity by directly binding to the TG or binding through an intracellular cofactor. A delicate balance between intracellular Ca^{2+} / Mg^{2+} in the milieu of the TG may be a mechanism for regulating the intracellular activity of this enzyme. Mg^{+2} ions have been reported as capable of partly replacing the Ca^{2+} requirement of partially purified liver TG.²⁸

The mol wt of the ABAE TG as determined by SDS-PAGE was ~5 kd lower than the guinea pig liver TG. The two proteins may be synthesized similarly but processed differently within the cells. Variability in the mol wt of the vascular TG could be due to intracellular proteases. Lacking amino acid sequences, we conclude that the two enzymes are similar but not identical.

The vascular tissues that contained the TG antigen included human aorta, renal capillaries, and hepatic arteries and veins, as well as arterioles and venules in the skin. Using immunohistochemistry, we found that the TG and fibronectin antigens codistribute along the sinusoids of human liver. At the light microscopic level, it is not possible to determine whether the sinusoidal localization of the TG is the result of its presence in sinusoidal endothelial cells, binding to the extracellular matrix, or its presence in the sinusoidal membranes of hepatocytes. Hepatocytes may also synthesize guinea pig liver TG but not have sufficient intracellular antigen to be detected by the monoclonal antibody. Either hepatic endothelial cells or hepatocytes may synthesize the protein and secrete it into the extracellular matrix where it

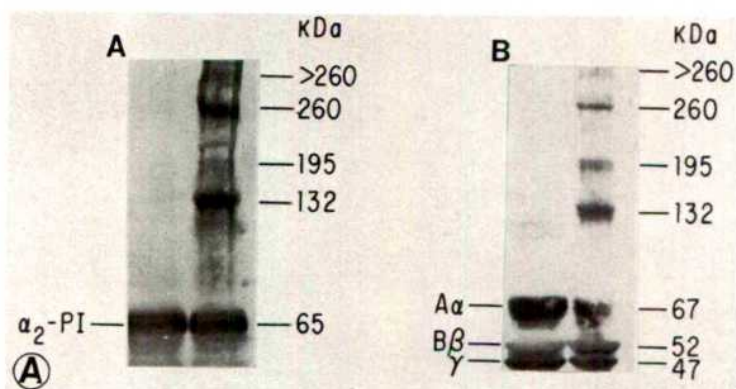


Fig 6A. Cross-linking of α_2 -plasmin inhibitor to fibrinogen by ABAE cell sonicates. ABAE cell sonicate was incubated with purified fibrinogen and α_2 -plasmin inhibitor in the presence of 5 mmol/L CaCl_2 or 5 mmol/L EDTA as described in the Materials and Methods section. Following SDS-PAGE and immunoblotting, the membranes were reacted with either anti- α_2 -plasmin inhibitor antisera (A) or anti-fibrinogen antisera (B), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin. Antigen bands were detected using a color reagent from BioRad. α_2 -Plasmin inhibitor migrated as a 65-kd protein band. The high-mol-wt complexes formed in the presence of 5 mmol/L CaCl_2 (mol wt = ≥ 132 kd) contain both α_2 -plasmin inhibitor antigen and fibrinogen antigen.

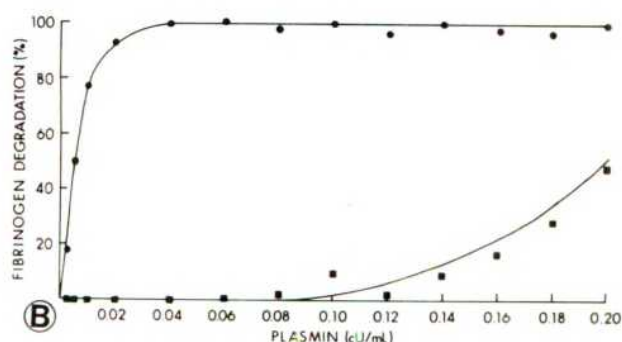


Fig 6B. Effect of ABAE cell lysates on fibrinogen degradation by plasmin. Plasmin was incubated with either fibrinogen alone (●—●) or with fibrinogen cross-linked to α_2 -plasmin inhibitor by ABAE cell lysates (■—■) as described in the Materials and Methods section. After plasmin treatment for 20 minutes at 37°C, the reaction mixture was solubilized and subjected to SDS-PAGE. Coomassie blue-stained fibrinogen bands were quantitated by scanning densitometry as described in the text.

may be bound to fibronectin. Fibronectin is a substrate for the plasma TG factor XIII_a^{4,25} and the tissue TG.²⁶ The monoclonal antibody used in this study does not cross-react with human factor XIII, fibrinogen, fibronectin, collagen, or bovine actin. Fibronectin is synthesized and secreted into extracellular tissues by vascular cells.²⁷ A similar mechanism may account for the TG distribution in vascular tissues. The immunohistochemical results represent the first demonstration of the in vivo distribution pattern of the TG antigen in human tissues. Preliminary electronmicroscopic immunohistochemistry studies suggested that the TG antigen was associated with the hepatic sinusoidal cells and not the hepatocytes (D. Bainton and C.S. Greenberg, unpublished observations).

The vascular TG described in this study is distinct from the plasma, TG factor XIII_a. Neither thrombin-dependent

TG activity nor factor XIII-related antigen was detected in the cells and tissue preparations used in the present study. Furthermore, the vascular TG (partially purified or crude cell sonicates) did not cross-react with polyclonal rabbit anti-factor XIII antibody. Finally, in contrast to γ -chain cross-linking of fibrinogen by factor XIII_a, the vascular TG preferentially cross-linked the α -chains to form dimers, mol wt = 132,000. Purified guinea pig liver TG also selectively cross-linked the α -chains of fibrinogen. These results demonstrate that the α -chain of fibrinogen is the preferred substrate for the ABAE cell and guinea pig liver TG. The ABAE cell TG also catalyzed the cross-linking of α_2 -plasmin inhibitor to the α -chains of fibrinogen. This is the first demonstration that a tissue-derived TG can function to cross-link α_2 -plasmin inhibitor to fibrinogen. The modified fibrinogen was nearly 40-fold more resistant to plasminolysis as compared with native fibrinogen. We have obtained similar results using the purified guinea pig liver TG (C.S. Greenberg and K.E. Achyuthan, unpublished observations).

The demonstration of a thrombin-independent TG in vascular cells and tissues that can utilize fibrinogen as a substrate provides a mechanism by which insoluble cross-linked fibrinogen could be deposited in normal and atherosclerotic vascular tissues.⁷ In addition, α_2 -plasmin inhibitor cross-linked to fibrinogen by the vascular TG might function to protect fibrinogen from plasmin degradation and then lead to accumulation of fibrinogen in the atherosclerotic plaques.⁷ The vascular TG could play an important role in fibrinolysis and could also provide an alternate pathway to promote fibrin stabilization following vascular injury.

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Antiidiotypic IgG Crossreactive With Rh Alloantibodies in Red Cell Autoimmunity

By S.P. Masouredis, M.J. Branks, and E.J. Victoria

IgG autoantibodies eluted from RBCs of antiglobulin positive normal blood donors contained at least two antibody populations, an IgG autoantibody (Ab 1), and an IgG population (Ab 2) that agglutinated RBCs coated with some Rh(D) alloantibodies. Eight of 24 autoantibody eluates tested agglutinated 3 of 10 anti-Rh(D) sensitized RBCs. The agglutinating activity was inhibited specifically by preincubation of the autoantibody eluate with the reactive anti-D. The reaction did not require the F_c domain of the anti-Rh(D), since autoantibody eluates agglutinated RBCs coated with F(ab')₂ prepared from the reactive anti-D sera. These findings indicate that the RBCs of some antiglobulin-positive blood donors contain an immunoglobulin auto-

antiidiotype (Ab 2) against the RBC autoantibody (Ab 1) which is demonstrable through its cross-reactivity with selected Rh(D) alloantibodies. Identification of auto-anti-idiotypes in RBC autoimmunity lends support to the idiotype-antiidiotype network hypothesis of immune regulation and is consistent with the bizarre and complex serology of autoimmune hemolytic anemia. The absence of clinical hemolysis in antiglobulin-positive normal blood donors suggests that immunoglobulin idiotype-antiidiotype interactions may play a role in modulating the effects of RBC autoimmunity.

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ANTISERA raised against relatively homogeneous antibodies or myeloma proteins contain a minor population of antibodies that react only with determinants in the antibody-combining region unique to the immunizing antibody, designated immunoglobulin idiotypes.¹⁻³ Rabbit antisera raised against isolated polyclonal or monoclonal Rh(D) antibodies contain idiotypic antibodies reacting with the immunizing Rh antibody which will cross-react with a limited number of unrelated Rh(D) antibodies.⁴⁻⁶ Interaction of both immunoglobulin and nonimmunoglobulin idiotypes expressed on lymphoid cells (T and B) are believed to control the immune response, as proposed by the network theory.^{7,8} Evidence that auto-anti-idiotopes are produced during the course of the normal immune response⁹ suggests that deranged immune regulation in autoimmunity may involve idiotype antiidiotype interactions.^{10,11}

In autoimmune hemolytic anemia (AIHA) of the warm type, the IgG autoantibodies (AAb) bind to RBCs optimally at 37 °C; most appear to be directed against an Rh-related determinant^{12,13} and lead to hemolysis and anemia.¹⁴ Rarely, either because of drug administration or without known cause, RBC autoantibodies arise that do not lead to hemolysis and such autoantibodies may occur infrequently in "normal" blood donors.^{15,16} Studies with radiolabeled autoantibodies have indicated that the IgG in such donor RBC

eluates contain other IgG populations in addition to the RBC autoantibody.¹⁷ One such class of IgG could represent an auto-antiidiotypic IgG (Ab 2) to the RBC autoantibody (Ab 1), which may be demonstrable through its cross-reactivity with idiotypes on Rh antibodies. This report shows that under certain conditions of storage and eluate preparation, IgG eluted from the RBCs of some IgG antiglobulin-positive normal blood donors contains auto-antiidiotypic-like reactivity that agglutinates RBCs coated with some alloanti-D sera.

MATERIALS AND METHODS

Isolation of RBC-bound IgG from antiglobulin-positive donor blood units. The principles in the Declaration of Helsinki were adhered to in this study, which was approved by the University Human Subjects Committee, and informed consent was obtained. The IgG antiglobulin-positive units used for these studies were obtained from the Los Angeles-Orange County American Red Cross Blood Services. All donors met the standard requirements for donating blood, and only the routine donor history was available. A negative drug history, particularly involving α -methyl dopa (α -MD), could not be verified retrospectively because of considerations of donor confidentiality and the effort required by the cooperating blood banks to track the donors many months after the donation. Some of the units may have been from donors on α -MD since this medication by itself does not exclude donors.

Eluates intended for autoantibody radiolabeling studies were prepared from >30 antiglobulin-positive donor units that had been collected and stored at 4°C for serologic studies by Dr George Garratty.¹⁸ As a result, some eluates used in these studies were derived from donor units that had been stored for up to 54 weeks after donor phlebotomy. The anti-IgG reactivity of the RBCs of the 24 donor units used for these studies varied. One unit had a 4+ reactivity, 7 units had a 1+, 12 units had a 3+ reaction, and 4 units were 2+. C3 was present along with IgG in ~70% of the units.

Eluates used for these studies were unlabeled and prepared from 24 donor units using two methods. Eluates from recently outdated units without significant hemolysis (10 units) were prepared by washing the RBC four times in 4 vol wash solution. Washes 1, 2, and 4 were in cold buffered saline, 0.15 mol/L NaCl, 0.015 mol/L phosphate buffer, pH 6.5 (BNS). The third wash was with high ionic strength 0.15 mol/L phosphate buffer, pH 6.5, $\mu = 0.26$ in an effort to remove nonimmune-bound IgG. Cell-bound IgG was eluted at acid pH using the Elukit Method (Gamma Biologicals, Houston, TX). The average storage time of units from which IgG was eluted from intact RBCs was 10.4 weeks from the time the donor was

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phlebotomized. An average of 0.7 mg IgG/U was recovered (range 0.04 to 3.2 mg).

Eluates from units with hemolysis were first converted to stroma by hypotonic lysis in an excess of 5 mmol/L sodium phosphate, pH 8.0 (5P8), followed by two washes with excess 5P8 and two additional washes with BNS. IgG was eluted from ghosts by adjusting the pH to 3.2 with 0.2 N HCl. The average storage time for units using ghosts for elution was 35 weeks from donor phlebotomy, with an averaged recovery of 6.4 mg IgG/U (range 0.003 to 27 mg).

The IgG-containing supernatant recovered from either ghosts or cells after centrifugation (1,000 g) was adjusted to pH 6.5 with 1 mol/L Tris, and the precipitate formed was removed by centrifugation. All eluates were equilibrated by dialysis with BNS containing 0.02% NaN₃ and 100 kallikrein inhibitor units (KIU) per milliliter of aprotinin (Trasylol, Mobay Chemical Corp, New York). The equilibrated IgG was heated at 56°C for 1 hour and centrifuged to remove any precipitate formed. Eluates that are not heat treated tend to form precipitates on standing whose composition has not been determined except that hemoglobin is present in the precipitate, possibly complexed to membrane proteins. This protocol is routinely used in the preparation of ¹²⁵I anti-D, and heating does not significantly reduce the anti-D content or the anti-D cell binding behavior. Presence of particulate matter in the eluates could result in nonspecific hemagglutination behavior. The eluates (~150 to 300 mL) were concentrated by Amicon XM-50 ultrafiltration to a final volume of 20 to 35 mL.

The eluates prepared from the 24 units used for these studies were obtained from RBCs washed four times. This washing protocol was adequate to remove the bulk of plasma proteins, as judged by the absence of anti-A activity in eluates derived from O or B donor units. IgG eluates obtained from units after only four washes were unsuitable for labeling studies. The labeled IgG did not demonstrate saturation binding and <20% of the labeled IgG was RBC adsorbable. Labeled RBC autoantibodies that demonstrated immunospecific binding required more vigorous washing (ten times) of the donor unit to dissociate and exclude IgG populations not involved directly in RBC binding.¹⁷

Characterization of RBC IgG eluates. Eluate IgG content was estimated by radial immunodiffusion using Ultra Low Level Tago Diffu-Gen Plates with a working range of 10 to 100 µg IgG/mL (Burlington, CA). The rabbit anti-human IgG (Gamma Biologicals) agglutination titer of the unconcentrated eluates ranged from 4 to 128, using a D-positive cell sensitized with doubling dilutions of the

IgG eluate. Agglutination was graded macroscopically (0 to 4+) by conventional serologic techniques. All the IgG eluates used in this study contained IgG RBC autoantibodies that would bind to common RBC as determined either serologically or after ¹²⁵I labeling of the eluted IgG.¹⁷

Serological methods. Eluates were tested for cross-reactive antiidiotypes after sensitizing D-positive RBC with individual donor anti-D sera (anti-IgG titers from 512 to 4,092). In a typical experiment, 200 µL of a 20% cell suspension was incubated with 200 µL of the anti-D serum for 1 hour at 37°C, followed by four washes with BNS. The sensitized RBCs were diluted with BNS to a final concentration of 3% to 5% and 50 µL of the sensitized cells incubated with an equal volume of AAb eluate for 10 minutes at RT. The cell suspension was centrifuged at 1,500 g for 15 seconds, and the cell pellet was graded for agglutination macroscopically.

Preparation of anti-D IgG fragments. F(ab')₂ anti-D fragments were prepared by solid-phase pepsin digestion of IgG¹⁹ previously isolated with protein A. Intact IgG and F_c fragments remaining in the supernatant were removed with protein A-Sepharose. The F(ab')₂ fraction was obtained after gel filtration using Sephacryl S-200 (Pharmacia, Piscataway, NJ). The presence of F(ab')₂ fragments was confirmed by sodium dodecyl sulfate (SDS) acrylamide electrophoresis, and the protein content was determined by the method of Lowry et al²⁰ with the use of bovine serum albumin (BSA) as a standard.

Antisera to IgG fragments. Rabbit anti-human IgG (Fc) and (F(ab')₂) and sheep anti-human IgG (F(ab')₂) were obtained from Cappel-Worthington, Malvern, PA. All three antisera were absorbed twice with an equal volume of packed human RBCs to remove saline agglutinating activity against normal human RBCs.

RESULTS

Autoantibody agglutination of anti-D coated RBCs. Table 1 shows the results obtained when 24 different AAb eluates were tested for their ability to agglutinate ten different anti-D-sensitized RBCs. Control unsensitized, BNS incubated RBCs did not agglutinate with the addition of any AAb eluate. Neither reactive nor unreactive AAb eluates had saline agglutinating activity for common (D positive or negative) or rare (Rh null or D- -) RBCs. Eight of the 24 AAb eluates agglutinated one or more anti-D-sensitized cells and only 3 of 10 anti-D sera tested were

Table 1. Agglutination of IgG Anti-D Sensitized RBCs by IgG Autoantibody Eluates

AAb Eluate No.	Autoantibody Eluate			AAB Agglutination of Anti-D-Sensitized RBCs				
	Anti-IgG Reactivity Donor's RBCs	Eluate Titer	Eluate IgG µg/mL	BNS-Incubated RBCs	Th	Bl	Sm	7 Other Anti-D Coats
6	3+	32	<3	0	1+	0	0	0
20	2+	32	1154	0	0	0	2+	0
21	2½+	64	37	0	0	1+	0	0
22	1½+	4	843	0	2+	±	2+	0
27	±	32	103	0	2+	0	0	0
32	2+	16	<3	0	1+	1+	0	0
34	3+	16	899	0	0	1+	0	0
35	4+	128	37	0	±	1+	0	0
16 Other eluates	variable	variable	variable	0	0	0	0	0
Anti-IgG-negative donor eluates (5)	0	0	0	0	0	0	0	0

The anti-D-coated D-positive RBC, R₁R₂ (CDe/cDe) sensitized with ten anti-D sera was from the same donor. All except one of the ten anti-D sera sensitized the D-positive RBC to produce a 4+ anti-IgG agglutination. The same RBC incubated in BNS did not agglutinate on addition of AAb eluate. In addition, a control D-negative RBC (rr) incubated with anti-D did not agglutinate on addition of AAb eluate. Sensitization and the autoantibody agglutination protocol are described in the text.

reactive. Reactive AABs, 6, 22 and 27 reproducibly agglutinated a reactive anti-D-coated cell when tested at three different times. Some autoantibodies, however, showed reduced activity with storage or when a different RBC was used. Reactivity was enhanced when both high-titered anti-D sera and high-density D site RBCs were used. Only one of the 10 anti-D sera gave less than a 4+ anti-IgG agglutination. Eluates prepared from five anti-IgG-negative donor units stored 9 to 10 weeks after phlebotomy had undetectable IgG levels and failed to agglutinate any of the ten anti-D-coated cells. The autoantibody eluates reactive with anti-D-sensitized RBCs had agglutination titers that ranged from 4 to 128, with an IgG content that varied from undetectable levels up to 1,154 μg IgG/mL. Immunospecificity of the reaction was supported by the following additional controls: D-negative RBCs incubated with the anti-D failed to agglutinate on addition of the AAB; RBCs sensitized with the AAB eluates failed to agglutinate on addition of two anti-D sera (Th and Sm), even though some of these AAB agglutinated anti-D (Th) sensitized RBCs; RBCs sensitized with 24 AAB eluates failed to agglutinate with the addition of the same AAB; and eight anti-D-coated RBCs failed to agglutinate with the addition of the same anti-D.

RBCs of different Rh phenotype coated with the same anti-D (Th) show significant variability in their agglutinability with the same autoantibody (AAB 22). Table 2 shows that there is both variability between different Rh phenotypes and within the same Rh phenotype. Four of 12 different R_1r anti-D-coated RBCs failed to agglutinate with the addition of AAB 22 and only four gave 1+ reactions.

No evidence was obtained in preliminary studies to indicate that AAB 22, which agglutinates (Th) anti-D-coated RBCs, cross-reacts with RBCs sensitized with either Rh alloantibodies or unrelated RBC alloantibodies. RBCs sensitized with a single example of anti-E, e, C, c, K, k, Fy^a , Fy^b , JK^a , and JK^b were not agglutinable by AAB 22. It will require testing of many individual serum samples of each antibody with many autoantibody eluates to establish conclusively that the antiidiotypic activity in autoantibodies is specific for only idiotopes on anti-D.

Table 2. Effect of Rh Phenotype of Anti-D-Sensitized RBCs on Autoantibody Agglutination

AAB 22 Agglutination of RBCs of Different Rh Phenotype Coated With (Th) Anti-D				
R_1r	R_1R_1	R_1R_2	R_2r	R_2R_2
O	1+	1+	1+	1+
$\pm$	$\pm$	2+	1+	1+
O	1+	O	$\pm$	—
$\pm$	1+	—	1+	—
$\pm$	2+	—	—	—
1+	1+	—	—	—
1+	1+	—	—	—
1+	1+	—	—	—
O	2+	—	—	—
1+	—	—	—	—
O	—	—	—	—
$\pm$	—	—	—	—

Results obtained with ten different R_1r , 9 R_1R_1 , 3 R_1R_2 , 4 R_2r , and two R_2R_2 and RBCs.

Neutralization of autoantibody antiidiotypic activity by reactive and unreactive anti-D sera. Preincubation of the AAB containing eluates 6, 22, and 27 with the reactive anti-D abolished the ability of the AAB eluate to agglutinate cells coated with that anti-D (Table 3). Equal volumes (50 μL) of AAB eluate and anti-D were incubated for 1 hour at RT before testing. AAB agglutination of the reactive anti-D coat (Th) was not inhibited by preincubation of the AAB with a nonreactive anti-D coat (Ov). The reduced reactivity of AAB 22 after preincubation with the nonreactive anti-D (Ov) probably results from the 50% dilution of the AAB during preincubation. The reduction from 2+ to $\pm$ in reactivity of AAB 27 following preincubation with the nonreactive anti-D (Ov) may, in addition to volume effects, be due to some degree of cross-reactivity sufficient to neutralize AAB 27 partially.

Agglutination of anti-D $F(ab')_2$ fragment coated red cells by autoantibodies. Red cells sensitized with $F(ab')_2$ fragments derived from a reactive anti-D were used to determine if the Fc domain is involved in AAB agglutination. Aliquots (20 μL of a 5% R_1R_2 (CDe/cDE) RBC suspension) were incubated with 100 μL of: BNS; (Th) IgG anti-D (9 mg/mL); (Th) anti-D $F(ab')_2$ fragments (7 mg/mL); and with (Ov) anti-D $F(ab')_2$ fragments (4.6 mg/mL) for 1 hour at 37°C. The sensitized cells were washed four times with BNS and then incubated with 50 μL of BNS, anti-IgG, anti-Fc, two anti- $F(ab')_2$ (sheep and rabbit), and AAB 22 for 10 minutes at RT. Following the second incubation, the cells were centrifuged at 1,500 g for 15 seconds and graded for agglutination.

Table 4 shows that AAB 22, which agglutinates RBCs coated with (Th) IgG anti-D, agglutinated RBCs coated with $F(ab')_2$ fragments derived from anti-D (Th). The presence of $F(ab')_2$ on the RBC was shown by agglutination of the $F(ab')_2$ -coated RBCs by anti- $F(ab')_2$ sera (both rabbit and sheep) and by failure of anti-Fc to agglutinate the $F(ab')_2$ coated RBC. RBCs coated with $F(ab')_2$ fragments prepared from an unreactive anti-D (Ov) remained inagglutinable when tested with AAB 22.

Preincubation of AAB 22 with either (Th) anti-D serum (Table 2, third column) or (Th) $F(ab')_2$ fragments (Table 3, last column) neutralized the ability of AAB 22 to agglutinate (Th) anti-D-sensitized RBCs. The conditions used for (Th)

Table 3. Anti-D Neutralization of Auto-Antiidiotypic Antibodies in RBC Eluates

Autoantibody Eluate No.	Agglutination of Anti-D (Th)-Coated RBCs by		
	AAB Alone, Control	AAB Preincubated With Anti-D (Th)	AAB Preincubated With Anti-D (Ov)
6	$\pm$	O	$\pm$
22	2+	O	1+
27	2+	O	$\pm$
Anti-IgG control	4+	4+	4+

A D-positive RBC, R_1R_2 (CDe/cDE) sensitized with anti-D (Th) was reacted with autoantibody eluate alone as control, reaction volume 100 μL (second column), with autoantibody eluate preincubated with anti-D (Th), reaction volume 150 μL (third column), and with autoantibody eluate preincubated with an unreactive anti-D (Ov) as control, reaction volume 150 μL (fourth column).

Table 4. Autoantibody 22 Agglutination of F(ab')₂ Anti-D Coated RBCs

RBCs Coated With	Agglutination Reaction With						AAb 22 Preincubated With (Th) F(ab') ₂
	BNS	Anti-IgG	Anti-Fc	Anti-F(ab') ₂ *	anti-F(ab') ₂ †	AAb 22	
IgG Anti-D (Th)	0	4+	3+	3+	3+	1+	0
F(ab') ₂ (Th)	0	3+	0	2+S	3+	1+	NT
F(ab') ₂ (Ov)	0	4+	0	2+	3+	0	NT
BNS	0	0	0	0	0	0	NT

Agglutination of a D-positive RBC, R₁R₂ (CDe/cDE) sensitized with IgG anti-D (Th) and (Th) anti-D F(ab')₂ fragments by antibodies to intact IgG, rabbit anti-human F(ab')₂*, sheep anti-human F(ab')₂† and AAb 22. The last column shows the effect of preincubating AAb 22 with Th F(ab')₂ on the ability of AAb 22 to agglutinate (Th) IgG anti-D-coated RBCs.

*Rabbit.

†Sheep.

F(ab')₂ inhibition were preincubation of 50 μ L AAb 22 with 75 μ L (Th) F(ab')₂ fragments (7.3 mg/mL) for 20 minutes at 37°C.

Rh serologic specificities of RBC autoantibodies. Autoantibody eluates sensitized common Rh(D)-positive and Rh(D)-negative RBCs to give variable anti-IgG agglutination consistent with the known serologic behavior of warm-type autoantibodies. Table 5 shows the anti-IgG agglutination of an Rh null (frozen-thawed) and an R₁R₂ RBC following incubation with either reactive or unreactive autoantibodies. Four of the eight reactive AABs bound strongly (3 to 4+) to Rh null RBCs, and all except one sensitized the R₁R₂ RBC. Cellano (k) reactivity served as an index of the effect of freezing and thawing on antigen expression using an unrelated Rh antigen. Rh antigen activity could not be directly evaluated, since even in the native state Rh null cells do not carry any Rh alloantigens. These serologic results are consistent with the binding of ¹²⁵I-labeled AAb to Rh null cells that show reduced and variable binding.²¹ No difference in the serologic activity of reactive or nonreactive AAb to Rh null RBCs was evident.

Influence of storage time and eluate IgG content on antiidiotypic activity. Table 6 shows the relationship between the ability of the AAb eluate to agglutinate anti-D-coated RBCs with the storage age of the unit before elution and the quantity of IgG recovered in the eluate. All AAb eluates were obtained from outdated units with storage times > 7 weeks. Neither IgG recovery nor storage time correlated with reactivity; both reactive and nonreactive

AABs had a similar pattern with respect to IgG recovery and storage age. No obvious association between eluates obtained from either RBCs cells or ghosts was evident. About 50% of the reactive eluates and 38% of the nonreactive eluates were derived from intact cells.

DISCUSSION

Some IgG antiglobulin-positive normal blood donors have a RBC-bound IgG population that appears to recognize idiotypic specificities on selected anti-D alloantibodies. This antiidiotypic IgG is probably an auto-antiidiotypic to the RBC autoantibody (Ab1) that shares idiotypes with some anti-D sera. Whether the anti-D idiotype is at the combining site and is unreactive following antigen binding (Ab 2 γ) or is removed from the combining site and unaffected by antigen binding (Ab 2 α) cannot be determined from these studies since the univalent binding of Rh antibodies and F(ab')₂ leaves a free Fab arm available to react.²² Other Rh alloantibodies or unrelated blood group antibodies do not show the cross-reactivity found with anti-D in preliminary studies using only one antiserum for each specificity. Failure to demonstrate cross-reactivity in hemagglutination is supported by inhibition studies of ¹²⁵I anti-D by either AAb or Rh antibodies. Some AABs inhibit the binding of anti-D, but Rh antibodies other than anti-D do not affect anti-D binding.²¹ Additional studies are needed to determine if other Rh-related antisera, such as anti-Rh 17 and 29, may carry more cross-reactive idiotypes than anti-D.

Identification of antiidiotypes in autoimmune hemolytic anemia has been reported in NZB mice.²³ Antiidiotypes to the RBC autoantibody in that study were available at high titers and could be demonstrated directly using hemagglutination techniques. Demonstration of antiidiotypes did not depend on cross-reactive idiotypes on related antibodies.

These studies all rely on hemagglutination, exquisitely sensitive but affected by many variables. Some of these appear to be the requirement for high-titered anti-D (>500), the use of a high D density RBC for anti-D coating, and the Rh phenotype of the anti-D-coated cell. Further work is needed to define the optimum conditions for detecting antiidiotypic activity, and efforts are underway to develop techniques that do not rely on hemagglutination for confirming these results.

It is evident that these studies have not defined the

Table 5. Autoantibody Binding to Rh Null and R₁R₂ RBCs

Autoantibody No.	Anti-IgG Agglutination of AAb Sensitized	
	Rh Null k-Positive	R ₁ R ₂ k-Positive
6	4+	4+
20	3+	3+
21	4+	4+
22	0	2+
27	3+	3+
32	±	±
34	±	3+
35	±	4+
Anti-k control	3+	3+
BNS control	0	0

Table 6. Storage Age and Quantity of IgG Recovered From Anti-IgG Positive Donor Units

No. of AAb Eluates	Total IgG Recovered per Unit μ g				No. of Weeks From Phlebotomy to Elution		
	0-50	50-100	100-500	>500	0-9	9-17	17-55
Reactive* eluates (8)	2	—	1	5	3	2	3
Nonreactive eluates (16)	5	3	1	7	3	4	9
Eluates from antiglobulin negative units (5)	5†	—	—	—	—	5	—

*Reactive AAb eluates were those that agglutinated one or more anti-D coats tested.

†IgG content <3 μ g.

optimum conditions for detecting antiidiotypic activity. Some of these factors probably include the effect of storage age, the role of washing, and the potential multiple interactions between autoantibodies and antiidiotypes, such as the extent and stability of complexes in the eluate. The role of long RBC storage time (>7 weeks) in the development of antiidiotypic activity is unclear. It is possible that prolonged contact of the AAb-coated cells with plasma enhances the binding of antiidiotypes to the AAb-coated RBCs and that the major source of the activity may be in the plasma and not on the RBCs. It also appears that the RBC-bound antiidiotypic activity is readily dissociated, since testing of a limited number of eluates obtained from exhaustively washed units (ten times) failed to demonstrate activity, which suggests that there may be a weak association of the antiidiotypic activity with the autoantibody.

The weak association between autoantibody (Ab 1) and antiidiotypic activity (Ab 2) provides an explanation for the ability of the eluate antiidiotypic activity (Ab 2) to be neutralized by anti-D and effect the agglutination of anti-D-coated RBCs. These effects would not be possible if the antiidiotypic activity had high affinity for Ab 1 and remained complexed to the autoantibody in the eluate. In these experiments, the ability of anti-D to compete for and neutralize dissociated Ab 2 would favor anti-D over Ab 1, since there was a large excess of anti-D with titers >512 as compared with autoantibody with titers <128.

The ability of Ab 2 to agglutinate anti-D-coated RBCs in the presence of eluate Ab 1 may be related to the experimental conditions used, which facilitated bivalent binding of Ab 2 to cell-bound anti-D. These conditions required a high cell-bound IgG density, achieved through the use of a high D density RBC and a high-titered anti-D, and centrifugal packing of the coated RBCs. As much as a 1,000-fold increase in affinity for Ab 2 would be expected following bivalent binding of Ab 2 with cell-bound anti-D.^{24,25}

The serologic reactivity of some of the autoantibody eluates with Rh null cells (Table 5) appears to be inconsistent with an Rh-related specificity of the autoantibody and would make cross-reactivity of the postulated antiidiotypic activity with anti-D-coated RBCs untenable. However, cross-adsorption and inhibition studies with ¹²⁵I-labeled autoantibody eluates indicate that AAb eluates contain multiple autoantibody populations both Rh and non-Rh-related.²¹ Those eluates reactive with Rh null RBCs do not have a single specificity. They contain in addition to non-Rh-related AAbs other autoantibody populations with Rh-related specificities.

Involvement of the idiotype-antiidiotypic network in human RBC autoimmunity introduces a level of complexity consistent with the known serologically bizarre behavior of RBC autoantibodies. If the antiidiotypic (Ab 2) is a type Ab 2 γ with the internal image of the antigen, the anti-antiidiotypic (Ab 3) may have specificity for the original antigen (Ab 3 = Ab 1). Such an interpretation would be consistent with the serologic demonstration of autoantibodies directed against alloantigens, ie, auto-anti-D, C, LW, En^a, etc. Such alloantigen specificities of autoantibodies on refined serologic analysis prove to be partial or incomplete and have been described as "pseudospecific."¹³ It would be expected that if Ab 3 is generated against the internal image of the antigen in Ab 2 γ that there would be a loss in fidelity consistent with the reported pseudospecificity of autoantibodies. Such an interpretation does not exclude the role of other factors in generating the serologic complexity of autoantibodies.

It was not possible to determine how many of the 24 normal blood donors used for these studies had RBC autoantibodies associated with α -MD administration. Almost uniformly, such IgG autoantibodies are not associated with cell-bound complement components.²⁶ The majority of the eight eluates (71%) that demonstrated antiidiotypic activity had cell-bound C3. An additional reason for identifying donors with α -MD autoantibodies is the reported effects of α -MD on immune regulation. α -MD in vitro was reported to inhibit T cell suppression of IgG production by pokeweed mitogen-stimulated B cells.²⁷ A recent attempt to confirm this finding was unsuccessful.²⁸ It therefore appears unlikely that the occurrence of antiidiotypic activity correlates in any simple fashion with autoantibody formation due to α -MD administration.

These results indicate that RBC eluates from some antiglobulin-positive normal blood donors contain antiidiotypic populations to the autoantibody. The potential clinical significance of this observation will depend on the ability to show that patients with autoimmune hemolysis either lack or have different antiidiotypic antibody populations. Such a proposed formulation will have to accommodate well-established factors associated with immune hemolysis such as quantity of cell-bound IgG and its subclass.^{29,30}

The participation of antiidiotypes in human autoimmune responses has been recently reported.³¹ These studies showed that recovery from anti-VIIIc (antihemophilic factor) autoimmune disease was associated with generation of antiidiotypes to the anti-VIIIc. A similar immune-modulating mechanism may be involved in antibody-mediated suppression of Rh alloimmunization³² and in the response of some

patients with chronic immune thrombocytopenia to high doses of immunoglobulin.³³ In both these situations, the modulation of alloimmune and autoimmune responses may be due to the presence of antiidiotypes in pooled Rh immunoglobulin and pooled normal immunoglobulin preparations.

Further studies are needed both to define the optimum conditions for demonstrating antiidiotypic activity and for isolating antiidiotypic antibody from the eluate possibly with a cross-reactive anti-D immunoadsorbant. A promising approach is the cloning of Epstein-Barr virus-transformed lymphocytes from Coombs-positive normal blood donors and

patients with hemolytic anemia. The generation by individual clones of monoclonal autoantibodies and antiidiotypes in sufficiently large quantities will simplify characterization of each type of antibody population and may provide material for therapeutic studies.

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Analysis of Hemoglobin F Production in Saudi Arabian Families With Sickle Cell Anemia

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Erythrocytes and progenitor-derived erythroblasts of sickle cell anemia patients from the Eastern Province of Saudi Arabia contain increased fetal hemoglobin and γ globin. A distinctive DNA polymorphism haplotype in the β globin gene cluster (+ + - + + + -), tightly coupled to a C $\rightarrow$ T substitution at position -158 5' to the cap site of the γ globin gene, is strongly associated with sickle cell disease in this region. To determine whether the increased fetal hemoglobin production and/or elevated γ globin content are tightly linked to this haplotype, we studied 55 members of five Saudi families in which sickle cell disease is present. The results did not suggest a tight linkage of the haplotype to increased fetal hemoglobin production. On the other hand, several sickle trait family members heterozygous for the haplotype had normal fetal hemoglobin pro-

duction in culture but elevated γ to α ratios in peripheral blood. This observation suggests that in this genetic background increased expression of the γ globin gene may occur without a measurable increase in total fetal hemoglobin production. The family studies also clearly demonstrate that increased fetal hemoglobin production by erythroid progenitors is dependent on zygosity for the sickle gene in this population. These findings strongly suggest that other factors, such as the products of genes stimulated by hemolytic stress or other genetic determinants associated with the Saudi β^S chromosome, may interact with the -158 C $\rightarrow$ T substitution and influence γ globin gene expression in this population.

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SICKLE CELL anemia (SCA) patients from the Eastern Province of Saudi Arabia have mean increased circulating fetal hemoglobin levels of $16\% \pm 7.4\%$, largely γ , whereas their sickle trait (AS) parents have mean levels in the normal adult range ($1.1\% \pm 1.0\%$).¹ Measurement of fetal hemoglobin in BFU-E-derived erythroblasts² in SCA patients and their AS parents provides strong evidence that the ability to produce increased HbF is inherited.^{1,3} Most Saudis with SCA are homozygous for a particular DNA polymorphism haplotype that characterizes the β globin gene cluster (+ + - + + + -).³ The γ -globin gene region of such a haplotype has been isolated by molecular cloning. Only one nucleotide substitution, a C $\rightarrow$ T at -158 5' to the γ cap site recognized by the restriction endonuclease *Xmn*I, was identified.³ The precise relevance of this substitution to the elevated HbF production in Saudi SCA is, however, uncertain since Saudi normals (AA) who may also have this

substitution do not demonstrate increased levels of fetal hemoglobin, either in circulating erythrocytes or in progenitor-derived erythroblasts.³

In an effort to examine the association of the -158 C $\rightarrow$ T substitution and high HbF expression in Saudis, we studied 55 members of five families with sickle cell disease. The results suggest that the -158 C $\rightarrow$ T substitution, although perhaps a necessary component of the elevated HbF program, is certainly not sufficient. Other factors, particularly those associated with homozygosity for β^S , appear at least equally important.

MATERIALS AND METHODS

Blood samples. Blood samples were obtained from Saudi Arabians who are employees of the Arabian American Oil Company (ARAMCO) and from their parents and siblings and children. Families were asked to volunteer blood samples of all available members after demonstration of elevated fetal hemoglobin in the blood of at least one member with SCA. SCA patients were not studied if they had undergone a blood transfusion in the past 6 weeks, had experienced a sickle cell crisis or active infection in the 7 days before assay, or were aged <3 years. The samples from Saudi Arabia were drawn in 10% acid-citrate-dextrose and shipped at 4°C. All assays and cultures were performed within 72 hours.

CBC and reticulocyte counts, hemoglobin electrophoresis on citrate agar and cellulose acetate, and alkali denaturation to determine percentage of fetal hemoglobin were performed on all samples. The ratio of γ/α in peripheral blood hemoglobin F was determined by high-performance liquid chromatography (HPLC)^{4,5} in samples in which a sufficient amount of hemoglobin F permitted this analysis.

Analysis of hemoglobin F in BFU-E-derived erythroblasts. Blood mononuclear cells were prepared and cultured in semisolid media with erythropoietin as previously described.¹ The culture plates were incubated in humidified 4% CO₂ at 37°C for 14 days, and erythroid colonies were then counted. These colonies were "plucked," and the average number of erythroid cells per colony was determined. A radioligand assay of total or fetal hemoglobin per BFU-E-derived cell was performed.^{1,6} Calculations of hemoglobin content per BFU-E-derived cell were based on the assumption that all such cells are potential F cells. Percentage of HbF was calculated from picogram of fetal hemoglobin and total hemoglobin content per cell. Reproducibility of this assay has been discussed previously but,

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in general, duplicate dishes or multiple assays performed on a sample from one individual yielded results within 10% of the mean.² Two Saudi sickle cell patients studied repetitively over 3 years in two different laboratories had mean percentages of HbF $\pm$ 1 SD in BFU-E-derived cells of $29.7\% \pm 3.8\%$ and $22.1\% \pm 3.2\%$.

Restriction endonuclease analysis of DNA and determination of the haplotype of the β globin gene cluster. Procedures for DNA isolation, blot hybridization, and probe preparations were as previously described.^{4,7} Restriction endonuclease site polymorphisms studied here included: an *XmnI* site 5' to the cap site of the $\epsilon\gamma$ globin gene, which recognizes the C $\rightarrow$ T substitution at position -158, an *AvaII* site in the IVS-2 of the β globin gene, and *HpaI* and *BamHI* sites 3' to the β globin gene. Digestion at the -158 *XmnI* site in Saudi sickle cell disease is always associated with the + + - + + 5' haplotype, representing polymorphisms at a potential *HincII* site 5' to the ϵ gene, a *HindIII* site in the IVS-2 of the $\epsilon\gamma$ globin gene, a *HindIII* site in the IVS-2 of the $\delta\gamma$ globin gene, and two *HincII* sites, one in the $\psi\beta I$ gene and another 3' to it.³ In this article, the presence of the "classic" 5' haplotype of the Saudi β globin gene complex (+ + - + +) was determined by digestion with *XmnI* at -158 C $\rightarrow$ T substitution and is denoted simply as +. The 3' haplotype described by the *Ava II* site in the IVS-2 of the β globin gene, the *HpaI* and *BamHI* sites 3' to the β globin gene, and the hemoglobin A or hemoglobin S genotype are fully noted. Thus, the complete classic Saudi β^S haplotype is described here as + + + - S.

RESULTS

Hemoglobin F production in five Saudi Arabian sickle cell families. Five Saudi Arabian families with sickle cell disease were studied to resolve whether determinants responsible for the increased HbF production observed in Saudis might be tightly linked to the β globin gene complex. Results of these studies are shown in Fig 1, families 1 through 5.

In family 1, all three children with SCA were homozygous for the + + + - S haplotype, which is frequently associated with Saudi sickle cell disease. They also had high $\epsilon\gamma/\delta\gamma$ ratios and increased, albeit variable, HbF production. One AS parent was homozygous (II-3) and the other heterozygous (II-2) for a -158 C $\rightarrow$ T substitution (*XmnI*-positive site), and both had increased HbF production. Two normal (AA) family members (I-1 and I-3) who were heterozygous for a -158 *XmnI* positive site and whose 3' β haplotypes were + + + rather than + + - did not have increased HbF programs, consistent with previous observations of normal HbF production in AA Saudis.³

In family 2, the SCA patient III-1 was heterozygous for a + + + - S β region haplotype and for another sickle chromosome for which the β region haplotype was - + - + S, a haplotype that is not associated with elevated hemoglobin F.^{3,8,9} The marked elevations of hemoglobin F, $\epsilon\gamma/\delta\gamma$ ratio, and HbF production in vitro observed in the SCA patient were all consistent with levels often observed in Saudi Arabian sickle cell disease. These data suggest that only one chromosome with the + + + - S β region haplotype is necessary to induce elevated HbF and high $\epsilon\gamma$ production in Saudi SCA. The paternal grandfather, I-4, who was heterozygous for the + + + - S β region haplotype also demonstrated increased HbF production in vitro. However, his son (II-3), who inherited the same sickle chromosome, did not. The patient's mother II-2 was heterozygous for - + - + S and + + + + A β region haplotypes.

Neither of these haplotypes would have been expected to be associated with elevated HbF production. Nevertheless, such an increase was clearly evident. The increased HbF production in culture was not itself due to the - + - + S haplotype because the patient's maternal grandmother (I-1) was heterozygous for this chromosome and did not have an increased HbF production. Similarly, the accompanying + + + + A haplotype was not itself responsible for the high HbF production in II-2 because its presence in I-2 was not associated with increased HbF. We must conclude that in II-2 the interaction of - + - + S and/or the + + + + A β regions with a factor(s) not detected in this analysis may have resulted in the high HbF production. This single family study strongly suggests that the + + + - S β region haplotype is not solely responsible for the increased HbF production observed in Saudis.

The three other family studies confirmed this impression. In family 3, the SCA patient (III-2) was homozygous for the classic Saudi + + + - S β region, but neither his circulating HbF nor percentage of HbF in BFU-E-derived cells was exceptionally increased. The remaining data in this family suggests that the two + + + - S containing chromosomes had different capacities for HbF production. The progenitor-derived erythroblasts with the maternal (II-2) + + + - S-containing chromosome had the expected increased HbF phenotype, whereas the paternal (II-3) phenotype was normal. This finding was recapitulated in the respective grandparents I-2 and I-5. That the + + + + A β region could, on rare occasions, also be associated with increased HbF production was suggested by II-6 and his daughter III-3. These two are the only exceptions that we have detected to the observation that high HbF production by BFU-E-derived erythroblasts in Saudis is only associated with β^S .

The results obtained from study of family 4 further emphasize the lack of tight linkage of the classic Saudi + + + - S β region to elevation of HbF production and circulating $\epsilon\gamma/\delta\gamma$ ratios. The proband (IV-1) was homozygous for the + + + - S β region haplotype and had very high circulating HbF and $\epsilon\gamma$ globin. His HbF production in vitro was substantially increased as well. Eight family members were studied, six of whom had sickle trait. All had at least one copy of the classic + + + - S β region haplotype. All of these had substantially higher hemoglobin F production in vitro than did the two family members with various β^A -containing haplotypes, but there was variability of both HbF production and percentage of $\epsilon\gamma$ in the sickle trait individuals. Again, this suggests that precise control of the HbF phenotype is not tightly linked to the particular haplotype involved. This impression was confirmed in family 5, in which the proband (III-1) had the expected elevated hemoglobin F, $\epsilon\gamma/\delta\gamma$ ratio and HbF production but was heterozygous for the classic + + + - S β region haplotype. His other β^S -containing chromosome - + - + S was, as expected, not associated with an elevated hemoglobin F production when found in the heterozygous state in other members of his family. The classic + + + - S β region haplotype present in his paternal lineage, while it produced a substantial elevation of $\epsilon\gamma$, was not associated with particularly high HbF production in vitro.

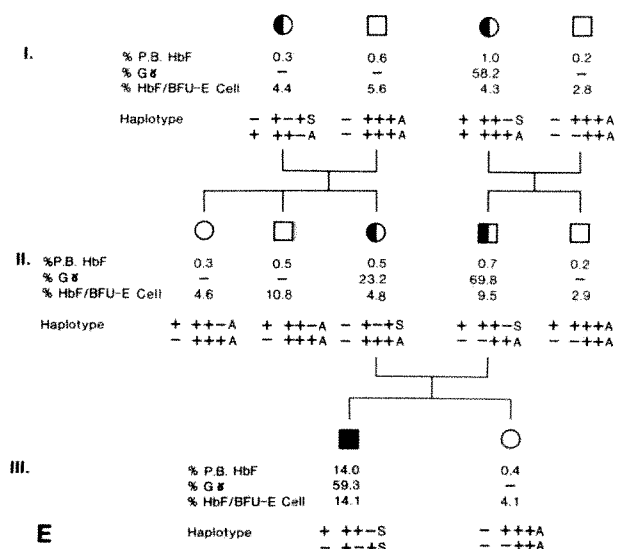
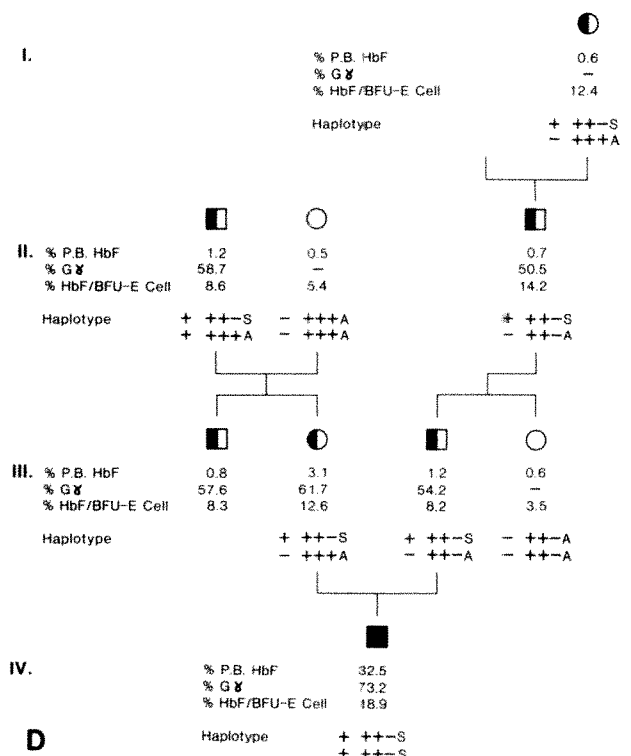
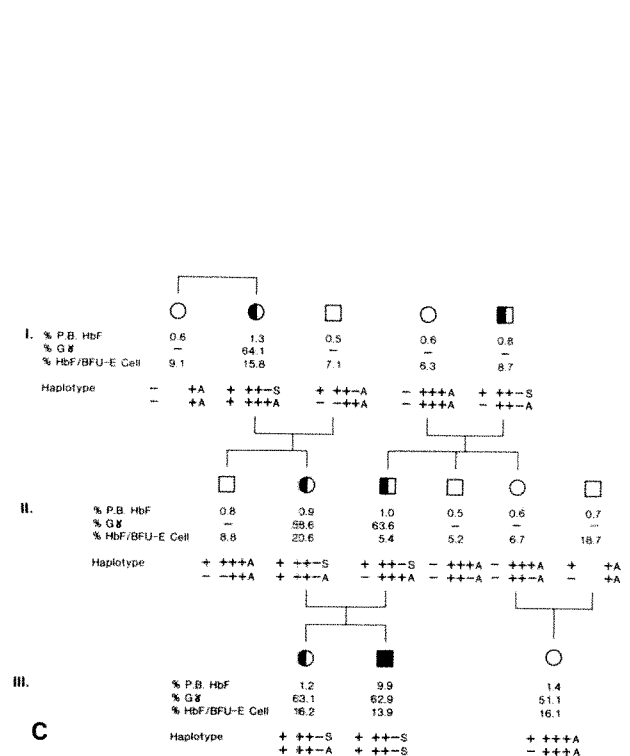
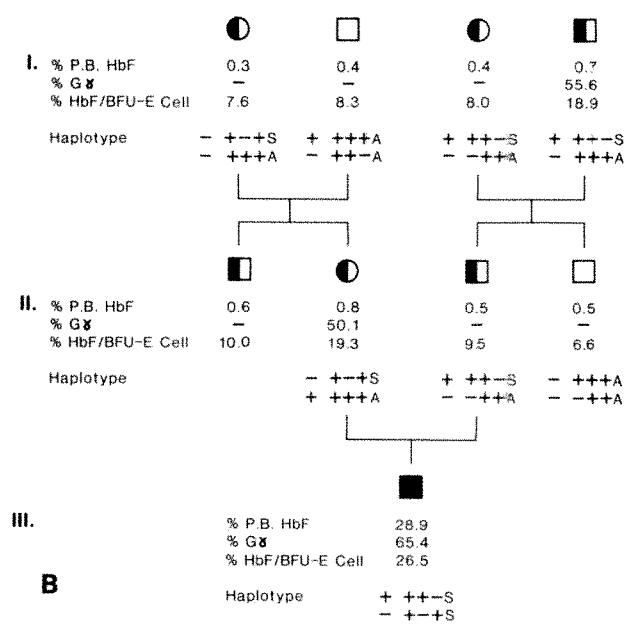
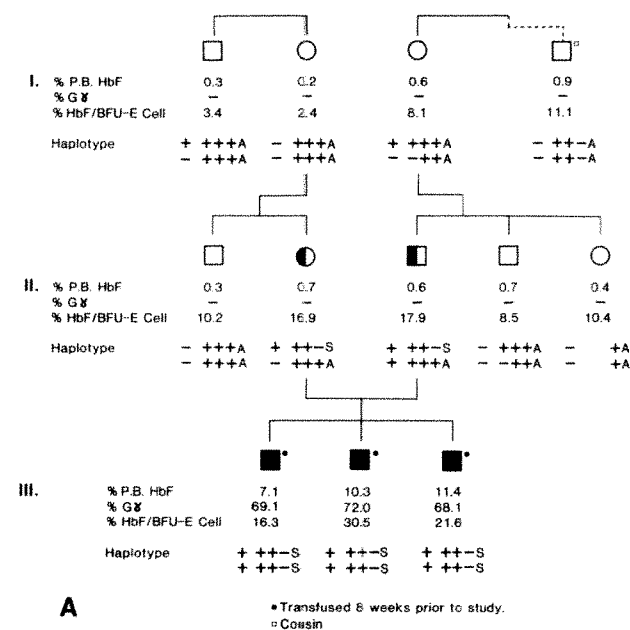


Fig 1. Analysis of percentage peripheral blood (PB) hemoglobin F, percentage of circulating $\alpha\gamma$ and percentage of hemoglobin F/BFU-E-derived cell in members of five families (families 1 through 5) with sickle cell anemia. Polymorphic restriction sites studied in the β globin gene cluster include, sequentially, the -158 *Xmn*I site 5' to $\alpha\gamma$, the *Ava*I site in the IVS-2 of the β globin gene, and the *Hpa*I and *Bam*HI site 3' to the β globin gene (described in the Materials and Methods section). Symbols: Male (□), female (○), AA (○, □), AS (●, ■), SS (●, ■). (A) Family 1; (B) Family 2; (C) Family 3; (D) Family 4; (E) Family 5.

Relationship of hemoglobin F production to zygosity for the Hb S gene. All members of these five families were grouped based on zygosity for the Hb S gene and the presence of the -158 *Xmn*I-positive site. The average percentage of HbF/BFU-E-derived cell for each individual is shown in Fig 2. There was no significant difference in hemoglobin F production between AA (-/-) and AA (+/-), between AS (+/-) and AS (+/+), or between SS (+/-) and SS (+/+). If more patients had been studied in several of these groups, significant differences between groups might have been demonstrated, but the differences would be small. As noted previously,³ significant differences were noted between AA (+/-) and SS (+/-) ($P < .01$), AS (+/-) and SS (+/-) ($P < .025$), and between AS (+/+) and SS (+/+) ($P < .05$). As a group, AA individuals had significantly lower hemoglobin F production in culture than that of AS individuals ($P < .025$), and AS individuals had significantly lower hemoglobin F production than that of SS ($P < .001$). Thus, the percentage HbF produced in culture appeared to be dependent on the degree of zygosity for the sickle gene. The -158 C $\rightarrow$ T substitution recognized by *Xmn*I cannot be solely responsible for increased fetal hemoglobin production, since AS (+/+) individuals produced significantly less fetal hemoglobin in culture and in blood than did SS (+/+). The SD in each group is large, suggesting multifactorial control of expression of γ globin genes.

Relationship of G_γ production to zygosity for the Hb S gene. Data on G_γ/Λ_γ ratios in these patients are incomplete because insufficient quantities of blood were available for isolation of hemoglobin F in all patients, particularly in those patients with <1% circulating fetal hemoglobin. G_γ/Λ_γ ratios are shown in Fig 3, together with the sickle phenotype and presence or absence of digestion by *Xmn*I at the -158 polymorphic site.

Saudis with AS or SS and a -158 C $\rightarrow$ T substitution, even when bearing this site on one chromosome, had an

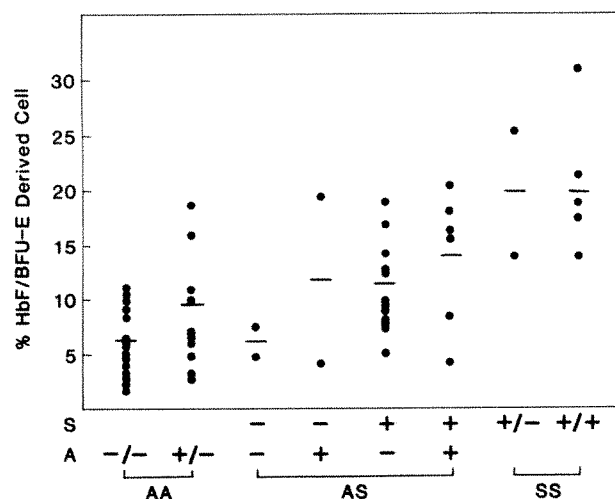


Fig 2. Assessment of percentage of HbF/BFU-E-derived erythroblast in AA, AS, and SS family members whose β globin gene clusters contain (+) or do not contain (-) the -158 C $\rightarrow$ T substitution 5' to G_γ . Bar indicates mean for each group.

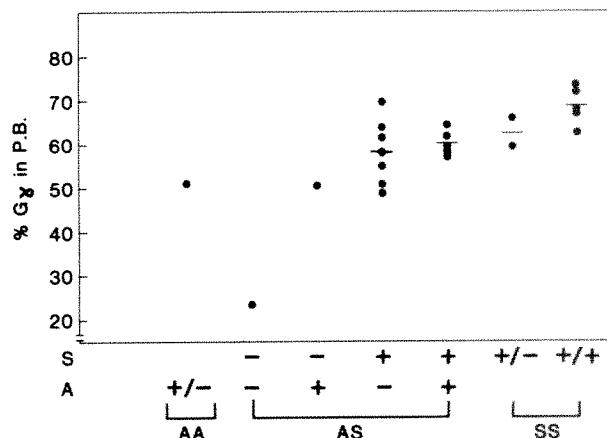


Fig 3. Assessment of the percentage of G_γ in the peripheral blood in AA, AS, and SS family members whose β globin gene clusters contain (+) or do not contain (-) the -158 C $\rightarrow$ T substitution 5' to G_γ . Bar indicates mean for each group.

elevated level of G_γ ($\geq 50\%$), suggesting that an increase in G_γ globin production can occur in association with this site without a measurable increase in total fetal hemoglobin production. No significant differences in G_γ production were noted in patients with AS (+/-) compared to AS (+/+), or in the two patients with SS (+/-) as compared to those with SS (+/+). However, individuals with AS (+/+) had significantly lower G_γ/Λ_γ ratios than did those with SS (+/+) ($P < .025$). G_γ/Λ_γ ratios were characterized by wide SD within a group, also suggesting that multiple factors control G_γ globin gene expression in these patients.

DISCUSSION

Sickle cell anemia is characterized by marked clinical diversity, which may partially reflect the different genetic backgrounds on which β^S arose.^{10,11} Increased hemoglobin F and/or G_γ production noted in some sickle cell and thalassemia patients has been associated with inheritance of a specific 5' haplotype of the β globin gene cluster (+ + + +) and with the presence of a C $\rightarrow$ T substitution at -158 5' to the G_γ globin gene.^{1,8,9,12,13}

We recently completed an analysis of Saudis from the Eastern Province with SS, AS, and AA genotypes, most of whom were at least heterozygous and often homozygous for the C $\rightarrow$ T substitution at -158 and β^{S3} . That analysis revealed an association of the C $\rightarrow$ T substitution with elevated HbF production and also showed that the β^S gene in these Saudis is usually linked to a distinctive 3' haplotype (+ + -). The present study of five Eastern Province Saudi families, in which there was at least one member with sickle cell anemia, was performed to determine whether the specific $\gamma\beta$ globin gene cluster frequently found in these individuals, could itself explain the high circulating hemoglobin F, increased G_γ/Λ_γ ratio, and elevated hemoglobin F production by progenitor-derived erythroblasts that characterizes these patients. Unlike Blacks and Mediterraneans heterozygous for deletion or nondeletion HPFH genes,¹⁴⁻¹⁶ Saudis with one copy of the "common" Eastern oasis haplotype, + + + -S do not have elevated total circulating HbF, although they do

tend to have variable increases in the $G\gamma/A\gamma$ ratio.^{1,3} To determine whether the $+++-S$ $\gamma\beta$ globin gene cluster is tightly linked to increase HbF production, we measured HbF production in the progenitor-derived erythroblasts of 55 family members of probands with sickle cell anemia and determined whether increased HbF production in these cells was associated with the presence of at least one copy of the $+++-S$ haplotype. We chose this approach because we had determined in previous studies that individual hemoglobin F production in culture is reproducible over relatively long periods of time and because the hemoglobin F production by BFU-E-derived erythroblasts in Saudis correlates with the total circulating HbF.^{1,2}

Five families provided an opportunity to study 11 different β^S -bearing chromosomes, nine of which had the $+++-S$ haplotype. We confirmed our previous finding³ that zygosity for the $+++-S$ $\gamma\beta$ haplotype correlates with the extent of HbF production in Saudis, consistent with the observation of other researchers that zygosity for HbS correlates with circulating HbF levels.¹⁷⁻¹⁹ However, the association does not

suggest tight linkage. Other mechanisms appear to be involved. For example, the $-158 C \rightarrow T$ substitution 5' to the $G\gamma$ gene of the $+++-S$ chromosome may simply increase the capacity of the $G\gamma$ region to respond to certain transacting regulatory proteins. The level of total HbF produced in erythroblasts might depend on the intracellular level of such proteins as well as on specific DNA sequences, both immediately 5' or at considerable distance from the γ globin genes.^{16,20-22} Erythropoietic stress, such as the hemolytic anemia of sickle cell disease, might affect *trans*-acting regulatory protein levels and thereby lead to increased expression of the $G\gamma$ genes and total hemoglobin F.

Clearly, ultimate understanding of regulatory mechanisms controlling high hemoglobin F production in Saudis and in other individuals with SCA such as Asian Indians,²³ awaits identification of the proteins that regulate globin gene expression and the development of *in vitro* expression systems that permit assessment of the function of such regulators in the presence of natural and artificial globin gene constructs.

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Extracellular Matrix of Cultured Bovine Aortic Endothelial Cells Contains Functionally Active Type 1 Plasminogen Activator Inhibitor

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The extracellular matrix (ECM) of cultured bovine aortic endothelial cells (BAEs) was analyzed by immunoblotting and reverse fibrin autography and shown to contain type 1 plasminogen activator inhibitor (PAI-1). Most PAI-1 in the ECM formed complexes with exogenously added tissue-type plasminogen activator (tPA), demonstrating that this PAI-1 was functionally active. The resulting tPA/PAI-1 complexes were recovered in the reaction solution, indicating that the PAI-1 in such complexes no longer bound to ECM. The PAI-1 could not be removed by incubating ECM in high salt (2 mol/L NaCl), sugars (1 mol/L galactose, 1 mol/L mannose), glycosaminoglycans (10 mmol/L heparin, 10 mmol/L dermatan sulfate), or ϵ -aminocaproic acid (0.1 mol/L). However, PAI-1 could be extracted from ECM by treatment with either arginine (0.5 mol/L) or potassium

thiocyanate (2 mol/L), or by incubation under acidic conditions (pH 2.5). ECM depleted of PAI-1 by acid extraction was able to bind both the active and latent forms of PAI-1. In this instance, most of the bound PAI-1 did not form complexes with tPA, indicating that the latent form was not activated as a consequence of binding to ECM. Although the PAI-1 activity in conditioned medium decayed with a half-life ($t_{1/2}$) of <3 hours, the $t_{1/2}$ of ECM-associated PAI-1 was >24 hours. These data suggest that PAI-1 is produced by cultured BAEs in an active form and is then either released into the medium where it is rapidly inactivated or into the subendothelium where it binds to ECM. The specific binding of PAI-1 to ECM protects it from this inactivation.

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THE REGULATION of the fibrinolytic system is of critical importance during hemostasis, wound repair, neoplasia, inflammation, and a variety of other biologic processes.^{1,2} It is becoming increasingly apparent that this control is achieved in large part through the action of specific plasminogen activator inhibitors (PAIs). Although four distinct PAIs have been detected,^{3,4} the endothelial cell-derived inhibitor (PAI-1)⁵ appears to be the physiologic inhibitor of tissue-type plasminogen activator (tPA). PAI-1 is a member of the serine proteinase inhibitor (Serpin) superfamily,⁶⁻⁸ and differs from other PAIs in its stability to sodium dodecyl sulfate (SDS),^{9,10} its electrophoretic mobility,¹⁰ and its ability to inhibit single-chain tPA.^{11,12} In addition, it appears to exist in at least two distinct forms in plasma¹⁰ and in media conditioned by cultured cells,^{13,14} including both an inherently active form and a latent form.^{13,15} Denaturants such as SDS or guanidine hydrochloride convert the latent PAI-1 into its active form.¹³ The physiologic mechanism for the activation of latent PAI-1 has not been delineated, but recent reports suggest that it is synthesized as an active molecule that is unstable and that rapidly decays into the latent form.^{15,16} In this report, we show that PAI-1 is present in the extracellular matrix (ECM) of cultured bovine aortic endothelial cells (BAEs). The ECM-associated PAI-1 is active and stable, and thus appears to be protected from inactivation.

MATERIALS AND METHODS

Reagents. All chemicals were the best analytic grade commercially available. Tissue culture materials were purchased from the following sources: Plasticware from Corning (Corning, NY); media from Flow Laboratories (McLean, VA); and calf serum, trypsin, penicillin, and streptomycin from GIBCO (Grand Island, NY). Materials were obtained as follows: bovine serum albumin (BSA), Triton X-100, Tris base, casein, α -D(+) mannose, β -D-galactose, dermatan sulfate, ϵ -aminocaproic acid (EACA), and EDTA from Sigma Chemical (St Louis); lysine-Sepharose and diethylaminoethyl (DEAE)-Sephadex from Pharmacia Fine Chemicals (Piscataway, NJ); L-arginine, glycine, and XRP-1 x-ray film from Eastman Kodak (Rochester, NY); lysine, Aprotinin, diisopropylfluorophosphate (DFP), and bovine fibrinogen from Calbiochem (La Jolla, CA); LPG-agarose and affinity-purified goat anti-rabbit IgG from

Miles Laboratories (Naperville, IL); Tween-80 from J.T. Baker Chemical (Phillipsburg, NJ); rabbit antiserum against bovine factor VIII from Biomedical Technologies (Stoughton, MA); biotin, streptavidin-alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, and 2-amino-2-methyl-propanediol from Zymed Laboratories (San Francisco); carrier-free Na ¹²⁵I from Amersham (Arlington Heights, IL); nitrocellulose sheets from Schleicher and Schuell (Keene, NH); 2-mercaptoethanol, solid-state lactoperoxidase-glucose oxidase and reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from BioRad Laboratories (Richmond, CA).

Fibrinolytic proteins. tPA was purified from human melanoma cell conditioned medium (CM),¹⁷ and its activity was determined by the ¹²⁵I-fibrin plate assay as described previously.¹⁸ The final product had a specific activity of ~500,000 IU/mg of protein according to the tPA International Standard and consisted primarily of the single-chain form. The tPA International standard (83/517) and the urokinase International Standard (66/46) were kindly supplied by the National Institute for Biological Standards and Controls (London). Rabbit antiserum to tPA was prepared by standard procedures, and purified goat antibodies to tPA were obtained from Bio-Pool (Hornefors, Sweden).

PAI-1 was purified from BAE CM by standard chromatographic techniques¹² without using SDS or other denaturing conditions. Analysis of the final preparation by SDS-PAGE and staining with Coomassie brilliant blue or periodic acid-Schiff revealed a single protein of mol wt 50,000. Antiserum to the purified PAI-1 was raised in New Zealand rabbits and was monospecific as determined by

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immunoprecipitation experiments and by two-dimensional gel electrophoresis.⁹ Monoclonal antibodies against the BAE PAI-1 were prepared as described.¹⁹ Purified latent PAI-1 was activated by incubation with SDS (0.1%) at 37°C for 60 minutes as described¹³ and then treated with Triton X-100 (1%) at 4°C for 1 hour to neutralize the SDS. Samples treated in this way were assayed for PAI-1 activity without further treatment or storage. tPA/PAI-1 complexes were prepared by mixing 1 µg tPA with 10 µg SDS-activated PAI-1, and the resulting complexes were purified by chromatography on lysine-Sepharose.²⁰ Native plasminogen was purified from human plasma by affinity chromatography with lysine-Sepharose followed by DEAE-Sephadex ion-exchange chromatography.²¹

Isolation of BAEs and preparation of CM and ECM. BAEs were isolated from bovine aortae and cultured in minimal essential medium (MEM) containing 10% calf serum.¹⁸ The cells used for these studies were cloned from a single factor VIII-positive cell.⁹ All cultures were grown to confluency in 60-mm dishes or in 24-well (16 mm each) culture plates and maintained at confluency for 3 days before use. To prepare CM, the monolayers were washed with serum-free MEM to remove the serum and then incubated in serum-free MEM for 4 or 24 hours. This CM was collected, centrifuged at 1,000 g to remove floating cells and cellular debris, and stored in 0.01% Tween-80 at -20°C until used. ECM was prepared from these or similarly treated cultures. The monolayers were washed three times with cold phosphate-buffered saline (PBS; 0.01 mol/L sodium phosphate, 0.14 mol/L NaCl, pH 7.4) and cellular components were extracted by incubation for 10 minutes with PBS containing 0.5% Triton X-100 at 37°C. The plates were washed an additional time with distilled water to remove remaining cellular components and then assessed by light-microscopic examination for the presence of cellular debris. These extraction procedures completely removed visible cellular components from dishes and plates.

Treatment of ECM. ECM was treated with various concentrations of tPA (0 to 200 ng/ml) in PBS containing 0.01% Tween-80, and 10 U/mL Aprotinin. After 1-hour incubation at 37°C, the reaction solution was removed and the dishes and plates were incubated for 30 minutes with PBS containing 5 mmol/L DFP to inactivate residual tPA. Dishes and plates were washed with PBS containing 0.05% Tween-80 and then with distilled water, and the ECM was extracted by scraping into 0.1% SDS. The extracts were then characterized as described below.

The ECM were also incubated with either 2 mol/L NaCl, 0.1 mol/L glycine, pH 2.5, or a variety of other reagents (Table 1). The pH of each of these solutions (except for the 0.1 mol/L glycine hydrochloride) was adjusted to pH 7.4 by adding 1 mol/L Tris, pH 7.4. After 2-hour incubation at 37°C, the plates were washed with solid-phase radioimmunoassay buffer (SPRIA buffer: PBS containing 1% BSA and 0.02% Tween-80), and the ECM was characterized.

Characterization of proteins in ECM. The proteins remaining in the ECM after various treatments were characterized by SDS-PAGE or by immunologic approaches. For the latter, the plates were incubated at 37°C for 2 hours with rabbit antiserum against either bovine PAI-1, bovine fibronectin, or bovine von Willebrand Factor (vWF), each diluted (1:500) in immunoradiometric assay buffer (IRMA buffer: PBS containing 3% BSA, 0.1% Tween 80, 20 U/mL Aprotinin, and 5 mmol/L EDTA). After the plates were washed, the bound antibodies were detected by incubating the plates at 37°C for 2 hours with ¹²⁵I-labeled goat anti-rabbit IgG in IRMA buffer. The plates were then washed sequentially with SPRIA buffer and distilled water. The bound antibodies were eluted from the ECM by incubating the plates for 30 minutes at 37°C in 1% SDS and were

Table 1. Effect of Various Treatments on PAI-1, vWF, and Fibronectin Content of ECM

Treatment	(Percentage of Control)		
	PAI-1	vWF	Fibronectin
NaCl 2 mol/L	100	—	—
β-D-galactose 1 mol/L	100	—	—
D-mannose 1 mol/L	100	—	—
Heparin 10 mg/mL	100	—	—
Dermatan sulfate 10 mg/mL	100	—	—
Lysine 1 mol/L	100	—	—
EACA 0.1 mol/L	100	100	100
Arginine 0.5 mol/L	59	100	100
Arginine 1 mol/L	41	100	95
Glycine, pH 2.5, 0.1 mol/L	27	100	96
Potassium thiocyanate 2 mol/L	42	—	75

BAE ECM were incubated with the indicated reagent for two hours at 37°C. The ECM were washed, and the PAI-1, vWF, and fibronectin content of the ECM was determined immunologically as described in the Materials and Methods section. Values are presented as a percentage of untreated controls incubated for two hours at 37°C in PBS.

counted in a γ-counter. A standard curve for the quantification of PAI-1 in ECM was constructed using purified PAI-1 coated directly onto empty plates. Purified PAI-1 (100 µL) at various concentrations in distilled water was added to 24-well culture plates, and the plates were incubated at 37°C for 16 hours. The plates were then incubated with 200 µL PBS containing 3% BSA at 37°C for 1 hour, washed extensively with SPRIA buffer, and then incubated with rabbit anti-bovine PAI-1 followed by ¹²⁵I-labeled goat antibody against rabbit IgG as described above. A similar approach was used to determine whether ECM treated in various ways was able to bind exogenously added PAI-1. Purified PAI-1 (100 µL) in IRMA buffer was added to ECM and after the plates were washed, was quantified as above.

SDS-PAGE. SDS-PAGE was performed in slab gels according to the procedures described by Laemmli.²² The upper stacking gel contained 4% acrylamide, and the lower separating gel contained 10% acrylamide. Samples were subjected to electrophoresis at room temperature for 16 hours at 50 V. After electrophoresis, the gels were stained with silver nitrate²³ or were analyzed for PAI-1 by immunoblotting (described below), or by either fibrin autography¹⁸ or reverse fibrin autography.²⁴ For fibrin autography, the formation of clear lytic zones in the opaque indicator film after 2 to 4 hours at 37°C reveals PA activity. For reverse fibrin autography, the development of opaque, lysis-resistant zones in the otherwise clear indicator film reveals PAI activity.

Immunoblotting. Samples were first subjected to SDS-PAGE, and then the proteins were electrophoretically (50 V, 1.5 hours) transferred to nitrocellulose using a buffer containing 50 mmol/L Tris base, 95 mmol/L glycine, 20% methanol, and 0.01% SDS.²⁵ The nitrocellulose sheets were soaked in PBS containing 1% casein (PBS/casein) for 1 hour at room temperature to block additional protein binding sites and then were incubated overnight at 4°C in PBS/casein containing the indicated antiserum (1:500 dilution). The nitrocellulose sheet was washed three times (10 minutes each wash) with PBS/casein and then incubated for 2 hours at room temperature with ¹²⁵I-labeled goat anti-rabbit IgG (250,000 cpm/mL). After being washed, the nitrocellulose sheets were dried and exposed to XRP-1 x-ray film for 48 hours at -70°C. To quantitate results obtained by immunoblotting, regions of the nitrocellulose sheets containing PAI were removed and counted in a γ-counter. These results were then compared directly to a standard curve

obtained by using known amounts of purified PAI-1, fractionated, and analyzed in parallel.

IRMA for tPA/PAI-1 complexes. Purified goat anti-tPA (50 μ L/well, 10 μ g/mL) in PBS was bound to U-bottom microtiter plates by incubation overnight at 4°C. The plates were then incubated for 1 hour in PBS containing 3% BSA to block any remaining reactive sites on the plastic and then were washed four times with SPRIA buffer. This washing step was included after each incubation step. Test samples and a standard curve of purified tPA/PAI-1 complexes were diluted in IRMA buffer and incubated (16 hours, 4°C) in the antibody-coated wells.²⁶ Bound tPA/PAI-1 complexes were quantitated by incubating each washed well first with rabbit anti-PAI-1 (1:750 dilution in IRMA buffer) and then with ¹²⁵I-labeled goat anti-rabbit IgG (10⁵ cpm/well). The wells were individually removed, and the radioactivity in each well was determined.²⁶

Enzyme-linked immunosorbent assay (ELISA) for bovine PAI-1. Purified monoclonal antibody (11B2) against BAE PAI-1 (100 μ L/well, 5 μ g/mL) in PBS was coated onto polystyrene microtiter plates by incubation at 4°C for 16 hours. The plates were then incubated in PBS containing 3% BSA at 37°C for 1 hour and then washed four times with SPRIA buffer. This washing step was included after each incubation step. Samples diluted in IRMA buffer were incubated in the antibody-coated wells at 4°C for 16 hours. Bound PAI-1 was quantitated by incubating each well first with biotin-labeled rabbit anti-PAI-1 antibody and then with streptavidin-alkaline phosphatase conjugate. Each well was incubated with alkaline phosphatase substrate (*p*-nitrophenyl phosphate) in 0.75 mol/L 2-amino-2-methyl-propanediol, pH 10.3, and the change in absorbance at 405 nm was analyzed using a Titertek multiscan plate reader.¹⁹

Miscellaneous. Protein concentration was determined by the method of Bradford.²⁷ Bovine fibrinogen was treated with DFP (1 mmol/L) before use. Bovine fibronectin was purified from bovine plasma by affinity chromatography with gelatin sepharose as described,²⁸ and rabbit anti-bovine fibronectin antiserum was provided by Dr C. Birdwell (La Jolla, CA). Purified human α -thrombin was a generous gift from Dr J. Fenton (Albany, NY). Antibodies were enzymatically labeled with ¹²⁵I by using solid-state lactoperoxidase-glucose oxidase reagents (Enzymobead, BioRad) and carrier-free NA ¹²⁵I. The ¹²⁵I-labeled goat anti-rabbit IgG had a specific activity of 2 to 5 $\times$ 10⁶ cpm/ μ g protein.

RESULTS

ECM-associated PAI-1 and its interaction with tPA. BAE ECM was extracted with SDS and consisted of a variety of proteins ranging in size from mol wt ~50,000 to mol wt >200,000 (Fig 1, lane 2). The smallest of these proteins appeared to comigrate with purified PAI-1 (lane 1). Analysis of the gel by reverse fibrin autorgraphy (lane 3) revealed that this protein exhibited inhibitory activity. Immunoblotting using monospecific antibodies to PAI-1 indicated that this inhibitor was PAI-1 (lane 4). Quantitative immunoblotting analysis demonstrated that these ECM extracts contained 2.5 to 5 ng/cm² PAI-1, representing ~10% to 20% of the protein present (data not shown). Similar amounts of PAI-1 were detected in the ECM of cultured human umbilical vein endothelial cells (data not shown).

We showed previously that most PAI-1 in CM is inactive but can be converted into an active form by SDS.¹³ To determine whether the PAI-1 of ECM was inherently active,

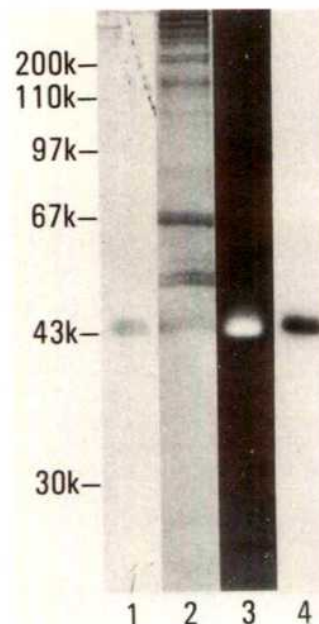


Fig 1. Analysis of the ECM of BAEs by SDS-PAGE. ECM prepared from three 60-mm culture dishes was extracted into SDS and then fractionated by SDS-PAGE under reducing conditions. The proteins present were detected by silver staining (lane 2), reverse fibrin autorgraphy (lane 3), and immunoblotting (lane 4). The position of purified PAI-1 (400 ng) is shown in lane 1 after silver staining.

or only active after exposure to SDS during extraction and electrophoresis, we evaluated the ability of untreated ECM to interact with exogenously added tPA. The ECM of BAEs were incubated for 1 hour at 37°C in the presence of increasing concentrations of tPA. The reaction solutions were removed, the ECM were extracted with SDS, and both solutions were then tested for PAI-1 activity by reverse fibrin autorgraphy (Fig 2). The addition of tPA caused a dose-dependent decrease in the PAI-1 activity of ECM with little detectable activity remaining in ECM treated with 100 to 200 ng/mL tPA (Fig 2A). Analysis of the reaction solution indicated that the decrease in PAI-1 activity in the ECM did not reflect a corresponding increase in PAI-1 activity in the medium (Fig 2B). A small amount of PAI-1 was spontaneously released when ECM was incubated in buffer alone (Fig 2B, lane 1). This spontaneously released PAI-1 activity also decreased in the presence of tPA.

To determine if the ECM-associated PAI-1 formed a complex with the exogenously added tPA, we examined the ECM extracts and reaction solutions by immunoblotting (Fig 3). The PAI-1 content of untreated ECM is shown in lane 1, and the positions of purified PAI-1 and of purified tPA/PAI-1 complexes are shown in lanes 4 and 5, respectively. Little detectable PAI-1 antigen remained in ECM treated with 200 ng/mL tPA (lane 2), most of it being recovered in the reaction mixture in complex with tPA (lane 3). Variable amounts of immunoreactive material (mol wt 43,000) also accumulated in the reaction solution and may

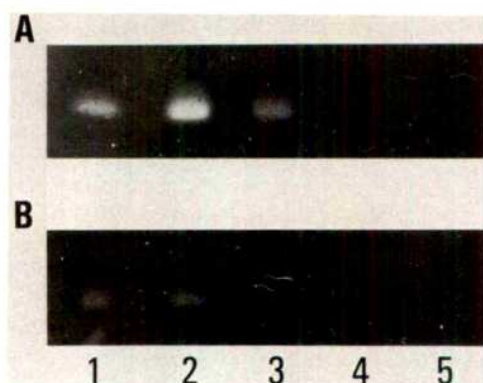


Fig 2. Effect of tPA on the PAI activity of ECM. ECM were prepared from 60-mm dishes incubated in the presence of increasing concentrations of tPA (1 mL) as described in the Materials and Methods section. The reaction solutions were removed, and the ECM were washed and extracted into SDS (1 mL). Aliquots (50 μ L) of the ECM extract (A) or reaction solution (B) were removed, treated with 0.5% 2-mercaptoethanol, and subjected to SDS-PAGE and reverse fibrin autography. Lane 1, 0 ng/mL tPA; lane 2, 10 ng/mL; lane 3, 50 ng/mL; lane 4, 100 ng/mL; and lane 5, 200 ng/mL.

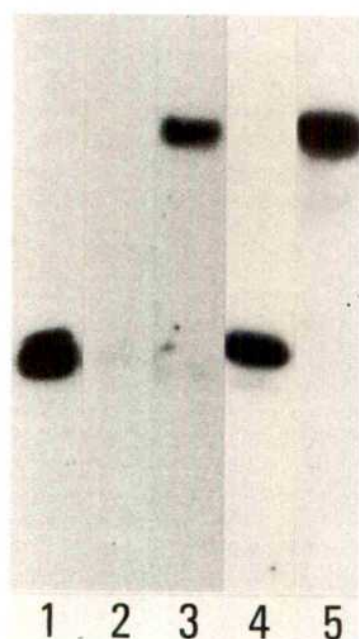


Fig 3. Effect of tPA on the PAI-1 antigen content of ECM. ECM prepared from 60-mm dishes were incubated in 1 mL PBS alone (lane 1) or in PBS containing 200 ng/mL tPA and 10 U/mL aprotinin (lanes 2 and 3). The reaction solutions were removed, and the ECM were washed and extracted into 1 mL 0.1% SDS. Equal aliquots (850 μ L) of the ECM extract (lanes 1 and 2) or reaction solution (lane 3) were removed, lyophilized, and subjected to fractionation by SDS-PAGE and analysis by immunoblotting using rabbit anti-PAI-1 as described in the Materials and Methods section. Lane 4 contains purified PAI-1 (100 ng) and lane 5 contains purified tPA/PAI-1 complex (100 ng).

represent cleaved PAI-1.²⁹ No tPA/PAI-1 complexes were detected in the ECM (lane 2).

An IRMA specific for tPA/PAI-1 complexes was used to quantitate the accumulation of these complexes in the reaction mixture (Fig 4). A dose-dependent increase of complexes was observed as the concentration of exogenously added tPA was increased. Approximately 30 ng/mL tPA/PAI-1 complexes were detected in the reaction solution when ECM was treated with 200 ng/mL tPA. The tPA in such complexes is resistant to DFP and can be detected after SDS-PAGE by fibrin autography.³⁰ The inset in Fig 4 (lane 1) shows purified tPA/PAI-1 complexes assayed for tPA activity in this way. Again, no complexes were detected in the ECM (lane 2), but they were present in the reaction mixture (lane 3).

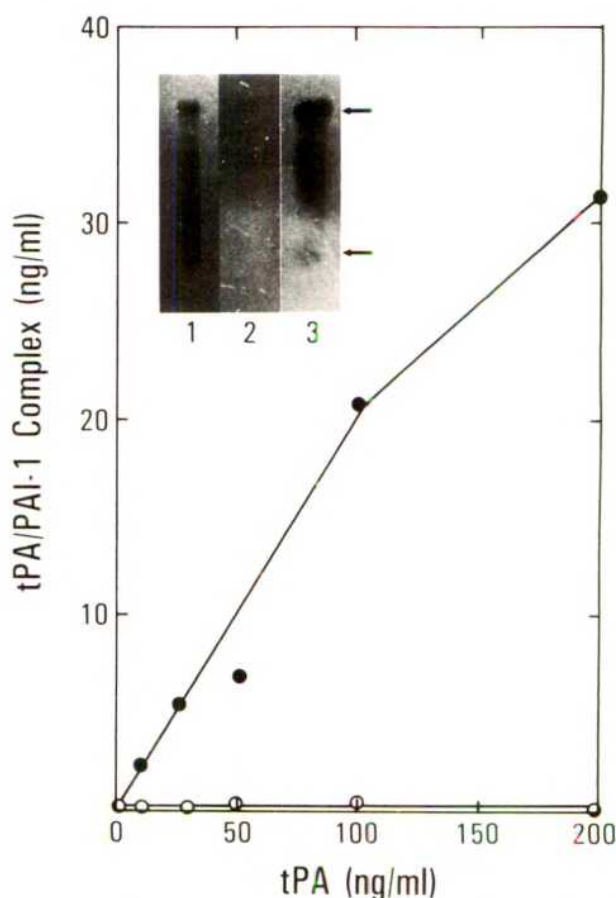


Fig 4. Quantitation of tPA/PAI-1 complex formation by IRMA assay. ECM prepared in 16-mm culture wells was incubated with 200 μ L of IRMA buffer in the presence of increasing concentrations of tPA in duplicate. After 1-hour incubation at 37°C, the reaction solution was harvested and treated with DFP and the ECM was washed and extracted into 200 μ L SDS (0.1%). Triton X-100 (1% final concentration) was added to the ECM extract to neutralize SDS. The concentration of tPA/PAI-1 complex in the reaction solutions ($\bullet$) and ECM extracts ($\circ$) was measured by the IRMA and was expressed as a tPA equivalent value. Inset shows complexes (upper arrow) revealed by fibrin autography; position of free tPA (lower arrow). Lane 1, purified tPA/PAI-1 complexes (10 ng); lane 2, extract from ECM treated with 100 ng/mL tPA; lane 3, 100 μ L reaction supernatant from ECM treated with 100 ng/mL tPA.

Nature of the association between PAI-1 and ECM. Experiments were performed to determine whether the association between PAI-1 and ECM represents a specific interaction (Table 1). BAE ECM were incubated with various reagents at 37°C for 2 hours. After washing, the PAI-1 remaining in the ECM was quantified immunologically as described in the Materials and Methods section. The PAI-1 content was not reduced when ECM was incubated in the presence of high concentrations of NaCl, mannose, galactose, heparin, dermatan sulfate, EACA, or lysine. However, the PAI-1 content was significantly reduced by incubation of ECM with arginine, potassium thiocyanate, and glycine (pH 2.5). These latter treatments only slightly decreased the concentration of fibronectin and vWF in ECM.

Analysis of the control and acid extracted ECM by SDS-PAGE and either reverse fibrin autography (Fig 5A, lanes 1 and 2), or immunoblotting (Fig 5B, lanes 1 and 2) confirmed the data shown in Table 1 and showed that acid treatment removed both PAI-1 activity and PAI-1 antigen. Experiments were performed to determine whether this treatment also removed the PAI-1 binding component of ECM. Acid-treated and washed ECM were incubated with purified PAI-1 for 24 hours at 37°C, washed, and extracted into SDS. The extracts were then tested for the presence of PAI-1. Both PAI-1 activity (Fig 5A, lane 3) and PAI-1 antigen (Fig 5B, lane 3) were present and could be detected

after SDS-PAGE. Thus, the acid-treated ECM appears to contain the PAI-1 binding component.

The purified PAI-1 used in these experiments consisted of both active PAI-1 (<5% of the total) and inactive PAI-1.^{12,13} The experiments shown in Fig 6 were conducted to determine whether both forms could bind to ECM and, if so, whether binding converted the inactive form into an active inhibitor. In these experiments, purified, non-SDS-treated PAI-1 was incubated with acid-treated ECM, and the ability of the bound PAI-1 to interact with exogenously added tPA was assessed. The added tPA did not decrease the amount of exogenous PAI-1 bound to acid-treated ECM (Fig 6, lanes 2 and 4), or result in the formation of detectable tPA/PAI-1 complexes (Fig 6, lane 5). These results are thus in contrast to the results with endogenous PAI-1 (Fig 2) and suggest that the latent PAI-1 was not converted into its active form on binding to ECM. The PAI activity detected in the ECM (Fig 6, lanes 1 and 2) results from the conversion of this latent PAI into its active form on exposure to SDS during extraction and/or SDS-PAGE.

Comparison of the stability of active PAI-1 in ECM and in CM. We showed previously that most PAI-1 in 24-hour CM is inactive,¹³ most likely because it is unstable and rapidly inactivated upon secretion.^{15,16} The demonstration that most PAI-1 in ECM was active (Figs 2 through 4) raised the possibility that PAI-1 bound to ECM had a longer half life ($t_{1/2}$) than that in CM. To test this possibility, the

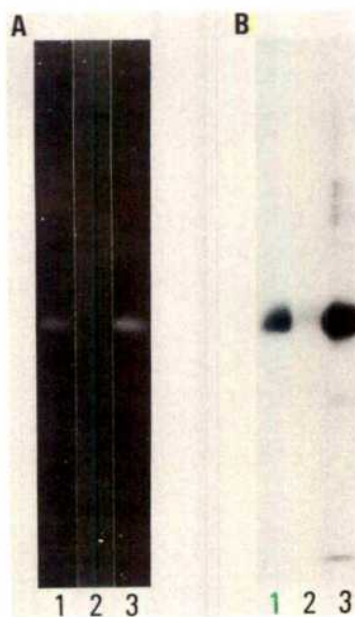


Fig 5. Binding of purified PAI-1 to acid-extracted ECM. ECM prepared on 60-mm dishes were incubated with 0.1 mol/L glycine, pH 2.5, at 37°C for 2 hours. This acid-treated ECM was then incubated with IRMA buffer in the presence of PAI-1 (1 μ g/mL) for 24 hours at 37°C. The ECM was then extensively washed, extracted into SDS, and subjected to SDS-PAGE. After electrophoresis, the gels were analyzed for PAI-1 activity by reverse fibrin autography (panel A) and for PAI-1 antigen by immunoblotting (panel B). Lane 1, extract of control, non-acid-treated ECM; lane 2, extract of acid-treated ECM; lane 3, extract of acid-treated ECM subsequently incubated with purified PAI-1.

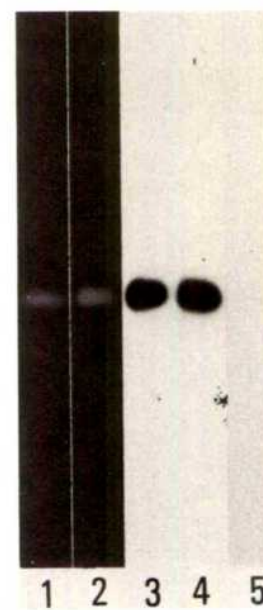


Fig 6. Effect of tPA on the PAI-1 bound to acid-extracted ECM. ECM prepared on 60-mm dishes were incubated with 0.1 mol/L glycine, pH 2.5, for 2 hours at 37°C. The resulting PAI-1-depleted ECM were then incubated for 24 hours at 37°C in IRMA buffer containing purified PAI-1 (1 μ g/mL). After extensive washing, the ECM were incubated in PBS (lanes 1 and 3) or tPA (200 ng/mL; lanes 2, 4, and 5) as described in the legend to Fig 2 and extracted into SDS. The extracts (lanes 1 through 4) and reaction supernatant (lane 5) were then analyzed for PAI activity by reverse fibrin autography (lanes 1 and 2) and for PAI-1 antigen by immunoblotting (lanes 3 through 5).

functional $t_{1/2}$ of ECM-bound and fluid-phase PAI-1 were compared (Fig 7). CM and ECM were incubated at 37°C for various times, and the remaining PAI-1 activity was determined. The PAI-1 activity of CM rapidly decayed, with an estimated half life of 3 hours (Fig 7A). The PAI-1 activity of ECM also decreased with time, but its apparent $t_{1/2}$ was 19 hours. PAI-1 antigen was spontaneously released from ECM on incubation (Fig 7B) and thus was not available for binding to tPA in these experiments. When this spontaneous loss of PAI-1 from ECM was taken into consideration (Fig 7A, solid circles), the ECM-associated PAI-1 appeared to be completely stable over a 24-hour period.

DISCUSSION

The ECM of cultured endothelial cells not only influences cell morphology, growth, and differentiation,³¹ but also binds and functionally alters components of the plasma fibrinolytic systems.^{32,33} The ECM consists of collagens,³⁴ proteoglycans,³⁵ and a variety of glycoproteins including fibronectin,³⁶ thrombospondin,³⁷ vWF,^{38,39} laminin,^{40,41} and a 47-kd protein.^{39,42,43} The n-terminal sequence of the 47-kd protein⁴³ is identical to that of human PAI-1.^{7,8} The data in this report

demonstrate directly that PAI-1 is a component of the ECM of endothelial cells (Figs 1 and 2), and as such may also influence endothelial cell physiology.

The PAI-1 is a specific ECM constituent and not a nonspecifically adsorbed cellular protein since the addition of ³⁵S-labeled PAI-1 to the cells during the isolation of ECM did not result in the binding of appreciable amounts of labeled PAI-1 to the ECM preparation (data not shown). More important, relatively harsh dissociating conditions, such as those that dissociate antibody/antigen interactions, were required to elute the PAI-1 from the ECM. For example, PAI-1 could not be removed from ECM by extraction with 2 mol/L NaCl (Table 1), suggesting that binding was not mediated by simple ionic interactions. The inability of heparin, dermatan sulfate, galactose, or mannose to elute PAI-1 suggests that neither glycosaminoglycans nor carbohydrate side chains are involved in the interaction between PAI-1 and ECM. Lysine residues may modulate the interaction between plasminogen and ECM.³³ However, these residues do not appear to be involved in PAI-1/ECM interactions since neither lysine itself nor EACA removes ECM-associated PAI-1. PAI-1 was only removed when ECM were extracted with acidic glycine, chaotropic agents, or arginine. The elution of PAI-1 from ECM by the basic amino acid arginine, but not lysine, suggests a specific role for arginine residues in this process. All treatments that removed PAI-1 from ECM did so without significantly decreasing ECM-associated fibronectin or vWF (Table 1). Thus, the binding of PAI-1 to BAE ECM, while strong and specific, differs from the binding of other matrix proteins.

PAI-1 is present in media conditioned by cells, in platelet releasates, and in blood^{3,4} in both an active and a latent form.^{10,13,15,44} The ratio of active to latent PAI-1 varies with cell type and culture conditions.^{45,46} The observation that these two forms are immunologically and biochemically similar,^{10,13,15,44} and that there is a single gene for PAI-1 in the human genome⁴⁷ suggests that the active and latent form are indeed two forms of the same molecule. Very little is known about which form is actually synthesized by cells or about the mechanism by which one form is converted into the other. The finding that the cell-associated form is active and rapidly converted into the latent form on release from the cell^{15,16} supports the hypothesis that PAI-1 is synthesized in the active, not the latent form. The data presented in this report are consistent with this hypothesis and may also provide clues about why PAI-1 is primarily active in some cells⁴⁵ and latent in others.^{13,15} For example, the ECM-associated PAI-1 from BAEs binds to, and forms specific, SDS-resistant complexes with tPA (Figs 2 through 4), indicating that it is primarily active. Latent PAI-1 does not bind to or interact with tPA.¹³ These results suggest that the interaction between PAI-1 and ECM influences PAI-1 activity. The observation that SDS and other protein denaturants convert the latent form into the active form¹³ initially suggested that the latent form was converted into the active form on binding. However, our results using purified PAI-1 do not support this hypothesis. For example, although exogenously added latent PAI-1 binds to ECM (Fig 6), the bound form still does not interact with tPA and thus does not appear

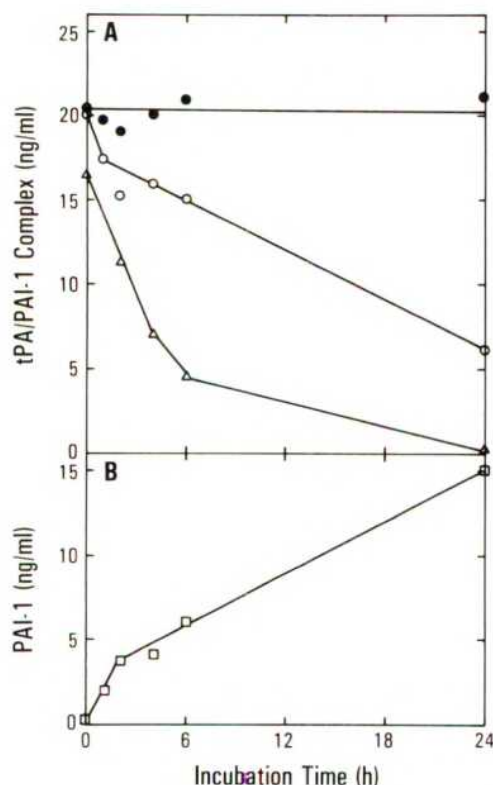


Fig 7. Stability of active PAI-1 in ECM and in CM. ECM were incubated in distilled water at 37°C for various times (panel B). The supernatants were removed, and their PAI-1 content (□—□) was determined by ELISA as described in the Materials and Methods section (panel A). The amount of active PAI-1 remaining in the ECM (○—○), or in 4-hour CM (△—△) incubated in parallel with ECM was also determined by measuring their ability to form complexes with exogenously added tPA (100 ng/mL) using the IRMA. The amount of active PAI-1 in ECM (●—●) after correction for the spontaneous loss of PAI-1 antigen shown in panel B.

to have been converted into its active form. Our results do not exclude the possibility that acid-extracted ECM, although still able to bind latent PAI-1, has lost the ability to convert it into its active form. Despite this, the data in Fig 7 imply a quite different role for ECM in this interaction. Thus, although the PAI-1 activity of CM decays with an estimated functional $t_{1/2}$ of 3 hours, the PAI-1 activity of ECM decreases with an apparent $t_{1/2}$ of 19 hours. In addition, when the data are corrected for the loss of PAI activity due to the spontaneous release of PAI-1 from the ECM (Fig 7B), the functional $t_{1/2}$ of the ECM-associated PAI-1 is considerably longer than 24 hours. These data suggest that the binding of active PAI-1 to ECM stabilizes it against the rapid decay that generally occurs when PAI-1 is released directly into the medium. Thus, PAI-1 is probably released from the cell in an active form and remains active when bound to ECM but rapidly decays in CM. The possibility that the active form detected in the cell¹⁵ and in CM from some cells⁴⁵ also reflects the presence of PAI-1 binding protein(s) that stabilize it remains to be explored. The recent demonstration of an active, but very high mol wt form of PAI-1 in plasma and cells^{10,46} is consistent with this idea.

The accumulation of tPA/PAI-1 complexes in the supernatant following exposure of the ECM to tPA suggests that the PAI-1 in such complexes has lost its ECM binding site. Little information is available concerning the nature of the ECM binding region of PAI-1. That inactive PAI-1 binds to ECM suggests that the reactive center of the molecule is not required. tPA cleaves the P₁-P_{1'} amino acids (Arg₃₅₇ Met₃₅₈) of the reactive center of PAI-1,⁴⁸ resulting in the formation of a C-terminal fragment (leaving group) of PAI-1^{7,48} of mol wt 3,000. The ECM binding site of PAI-1 may itself be

contained within this C-terminal fragment. Alternatively, cleavage of PAI-1 by tPA and the subsequent formation of tPA/PAI-1 complexes may induce a conformational change in PAI-1, resulting in its decreased binding affinity for ECM. The nature of the binding component in ECM also remains to be determined.

The extracellular matrix of endothelial cells binds several proteins involved in blood coagulation and fibrinolysis, including vWF,^{38,39} plasminogen,³³ fibronectin³⁴ and, as detailed in this study, PAI-1. When the endothelial cell lining is disrupted, blood coagulation is initiated and a fibrin clot is formed in association with the subendothelial matrix. In view of the results we present, active PAI-1 bound to the matrix may be expected to play a critical role in protecting the ECM and the ECM-associated fibrin from PA-mediated degradation through its specific inactivation of PAs. Furthermore, active PAI-1 released from platelets on blood coagulation,^{3,4,49} may also bind to the ECM, thus providing additional protection from fibrinolysis. Plasminogen activation provides an important source of localized proteolytic activity not only during fibrinolysis but also during ovulation, cell migration, epithelial differentiation, tumor invasion, and a variety of other physiologic processes.^{50,51} Many of these events involve movement of cells through ECM. The presence of PAI-1 in the ECM may thus influence a variety of biologic events.

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An α -Globin Gene Initiation Codon Mutation in a Black Family With HbH Disease

By Nancy F. Olivieri, Lebe S. Chang, Annette O. Poon, Alan M. Michelson, and Stuart H. Orkin

The molecular basis of hemoglobin H disease in a Black family of Canadian origin was investigated. Affected individuals had a combination of deletion and nondeletion α -thalassemia mutations on different chromosomes. Cloning and sequencing of the DNA of one member with the nondeletion form revealed a new thalassemia mutation, an

A $\rightarrow$ G substitution, in the initiation codon of the remaining α -globin gene of a rightward ($-\alpha^{3.7}$) deletion chromosome. This mutation abolished an *NcoI* restriction site and therefore is detectable in genomic DNA by Southern blot analysis.

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THE HUMAN α -globin gene complex on chromosome 16 consists of two adult α -genes ($\alpha 1$ and $\alpha 2$), an embryonic ζ -gene, and associated pseudogenes.¹ Disorders of α -chain synthesis result in diminished (α^+ thalassemia) or absent (α^0 thalassemia) α -chain production from the affected chromosome. Molecular analysis has revealed deletion and nondeletion defects of which, unlike the β -thalassemias, deletion mutations are the most common.²

Four clinical syndromes of α -thalassemia are recognized: the silent carrier, characterized by the presence of three functional α -globin genes ($\alpha\alpha/-\alpha$); α -thalassemia trait, by the presence of two α -genes ($--/\alpha\alpha$) or ($-\alpha/-\alpha$); Hb H disease, by the presence of one α -gene ($--/-\alpha$); and hydrops fetalis in which no functional α -globin genes are present ($--/--$).³ Varying extents of DNA deletions are responsible for these disorders.⁴⁻¹⁰

A less common class of α -thalassemia occurs in which the α -globin structural genes are grossly intact but contain mutations affecting RNA processing, RNA stability, and polyadenylation.¹³⁻²² Two mutations involving the initiation codon of the α -globin gene have been described. A dinucleotide deletion preceding the ATG initiation codon of an α -globin gene in an Algerian patient^{23,24} was responsible for a 30% to 50% reduction in translation efficiency of the α -globin RNA.²² A mutation within the initiation codon (ATG $\rightarrow$ ACG) of the $\alpha 2$ gene of a Sardinian patient abolished the function of this gene at the level of translation.²⁵ In a single Sardinian individual, the *NcoI* restriction site in an $\alpha 1$ -globin gene was abolished by a mutation that was not characterized at the DNA sequence level but was presumed to be the same.²⁶ We report here the first mutation within the initiation codon of an α -globin gene in a Black individual.

MATERIALS AND METHODS

Collection and analysis of blood specimens. Approximately 20 mL of blood from all patients of family G was collected in vacutainers with EDTA as anticoagulant. Hematologic and hemoglobin studies were carried out within 24 hours. Blood for hemoglobin chain synthesis was processed within 2 hours after collection. DNA preparation from blood samples was performed within 24 hours.

Hematologic and hemoglobin studies. Routine hematologic data were obtained with a Coulter counter model S-plus 4 with QC data terminal. Other hematologic data were obtained using standard laboratory procedures.²⁷ Iron status was evaluated with ferritin levels.²⁸ Hemoglobin studies included starch gel electrophoresis at pH 9.0 for the detection of abnormal hemoglobins,²⁹ diethylaminoethyl (DEAE)-cellulose microchromatography for the quantitation of Hb A₂,³⁰ and alkali denaturation for the quantitation of HbF.³¹ Hemoglobin chain synthesis was carried out as previously

described.³² HbH detection was performed using brilliant cresyl blue. DNA isolation and blot hybridization studies were performed as previously described³³ using restriction enzymes *Bam*HI and *Bgl*II. The ³²P-labeled probe used for these studies was total plasmid JW101.³⁴ For DNA cloning, total genomic DNA of the daughter (II-1) was digested to completion with *Bam*HI, ligated without size fractionation to the arms of EMBL 3 vector and packaged in vitro.³⁵ Recombinant phage were propagated in *Escherichia coli* and a phage clone containing the 10.5-kb fragment containing the putative nondeletion α^{TH} gene was identified by hybridization with a ³²P-labeled specific probe (1.5-kb *Pst*I fragment from the genomic subclone pRB $\alpha 1^{35}$). The 1.5-kb *Pst*I fragment containing this gene was subcloned in plasmid PUC 19. The recombinant plasmid containing the *Pst*I fragment containing the α^{TH} gene was digested with *Pst*I and *Hind*III and subcloned into M13mp18 and M13mp19 for DNA sequencing by the dideoxy chain termination method.³⁷ The sequence from the *Pst*I site 450 nucleotides 5' to the cap site to the *Pst*I site 3' to the gene was determined. Sequencing of both strands was performed for the *Hind*III site in exon-2, from the 5' and 3' flanking *Pst*I sites, and finally from a *Nae*I site 270 nucleotides 5' to the gene to the *Hind*III site.

RESULTS

The family studied (Fig 1) presented with the clinical and hematologic findings of HbH disease in the two younger children (II-2 and II-3, Fig 1 and Table 1). Digestion of genomic DNA of these patients with *Bam*HI and hybridization with α -globin gene probe revealed the presence of a 10.5-kb fragment, and digestion with *Bgl*II yielded a 16-kb fragment (Fig 2). This pattern is consistent with either of two genotypes: $--/-\alpha^{3.7}$ or $-\alpha^{3.7}/-\alpha^{3.7}$.

Hematologic and DNA findings in the mother were consistent with the silent carrier state of α -thalassemia. *Bam*HI

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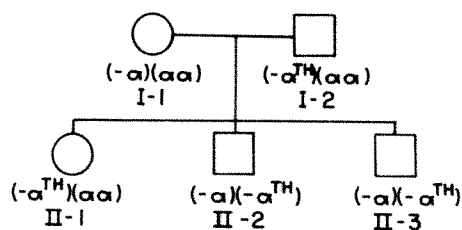


Fig 1. Pedigree and α -globin genotypes of family G.

digestion of genomic DNA and hybridization with the α -probe yielded fragments of 14 and 10.5 kb, whereas *Bgl*II digestion yielded fragments of 16, 12, and 7 kb, consistent with the genotype $\alpha\alpha/-\alpha^{3.7}$ (rightward deletion). Review of the father's hematology, however, revealed more marked microcytosis, occasional HbH in erythrocytes incubated with BCB, and a slightly more abnormal $\alpha/\beta+\gamma$ -globin chain ratio consistent with α -thalassemia trait. DNA analysis in the father also demonstrated fragments of 14 with 10.5 kb with *Bam*HI digestion and 16, 12, and 7 kb with *Bgl*II digestion. This pattern was consistent with two α -genes on one chromosome and one α -gene on the other in the arrangement $\alpha\alpha/-\alpha^{3.7}$. However, the presence of two children with HbH disease suggested that one other α -gene in the father was present but nonfunctional and that it resided on the 10.5-kb fragment. This conclusion was supported by studies in the daughter (II-1), which suggested that she had inherited two functional genes from her mother (on the 14-kb band on *Bam* digestion) and a single dysfunctional α -gene (on the 10.5-kb fragment on *Bam*HI digestion) from her father.

The 10.5-kb fragment carrying the putative nonfunctional gene was cloned and sequenced. The DNA sequence of this single α -gene was entirely $\alpha 1$ in type, as it contained an *Apa*I site in the second intervening sequence (IVS-2) and its 3' end was identical with that of a normal $\alpha 1$ gene.³⁸ The gene displayed a single mutation (ATG $\rightarrow$ GTG) in the initiation codon.

Southern blot analysis. Double digestion of genomic DNA with *Pst*I and *Nco*I and hybridization with α -probe produces 0.9-kb and 0.6-kb fragments in normal individuals. Because the mutation ATG $\rightarrow$ GTG abolishes the *Nco*I site cleavage recognition (CCATGG) within this *Pst*I fragment, digestion with *Pst*I and *Nco*I should yield a 1.5-kb fragment in all family members with the nondeletion α -thalassemia determinant. This prediction was verified experimentally, as shown in Fig 3.

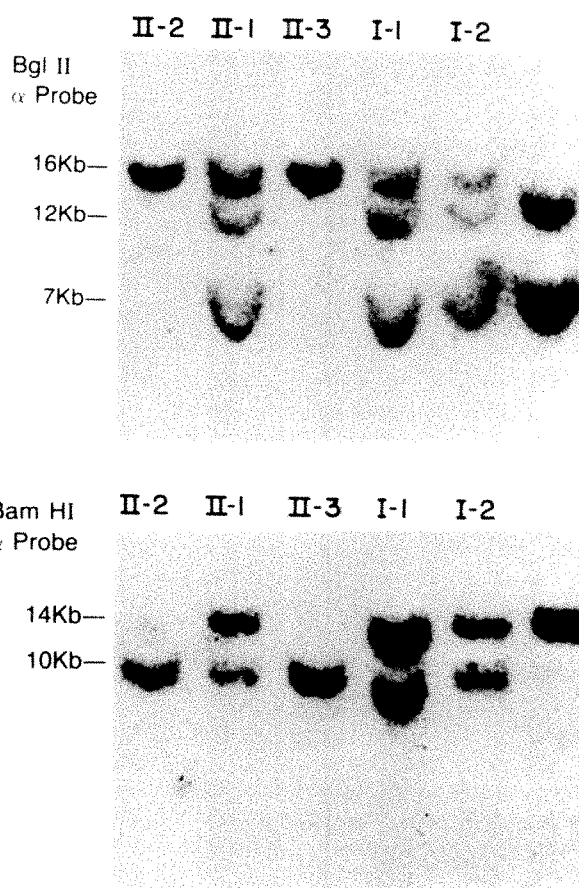


Fig 2. Southern blot analysis of family G.

DISCUSSION

We investigated the molecular basis of nondeletion α -thalassemia in a Canadian Black family. Although genetic defects leading to α -thalassemia have been extensively studied in Mediterranean populations, little is known regarding specific lesions in Black individuals with this form of the disorder. In the family we examined, two patients homozygous for the rightward ($-\alpha^{3.7}$) deletion and affected with HbH disease bore a single base substitution in the translation initiation codon of one of their remaining α -globin genes. This mutant gene was carried on a $-\alpha^{3.7}$ chromosome, the result of a crossover event between chromosomes bearing duplicated α -globin genes.^{2,38,39} Because the sequence of this gene was otherwise identical to that of a normal $\alpha 1$ -globin gene, we infer that the defect either arose originally within

Table 1. Clinical and Hematologic Findings in Family G

Subject	Hb(g/dL)	RBC	MCV(fL)	Hprep	$\alpha/\beta+\gamma$ Ratio Chain	A	Hb Elect				Fragments		Proposed Phenotype	Proposed Genotype
							F	A ₂	H	Barts	<i>Bam</i> HI (kb)	<i>Bgl</i> II (kb)		
I-1	15.0	5.6	81	—	.92	98.3	0.2	1.5	—	—	14,10	16,12,7	Silent carrier	($-\alpha$)($\alpha\alpha$)
I-2	14.6	6.8	71	OCC+	.87	98.6	0.2	1.6	—	—	14,10	16,12,7	Thal trait	($-\alpha^{TH}$)($\alpha\alpha$)
II-1	11.9	6.2	67	OCC+	.87	98.1	0.3	1.6	—	—	14,10	16,12,7	Thal trait	($-\alpha^{TH}$)($\alpha\alpha$)
II-2	9.4	6.1	51	4+	.41	89.1	4.7	1.0	2.4	2.1	10	16	Hb H disease	($-\alpha^{TH}$)($-\alpha$)
II-3	9.9	6.1	54	4+	.43	88.7	2.3	0.4	7.2	1.4	10	16	Hb H disease	($-\alpha^{TH}$)($-\alpha$)
Normal											14	12,7		($\alpha\alpha$)($\alpha\alpha$)

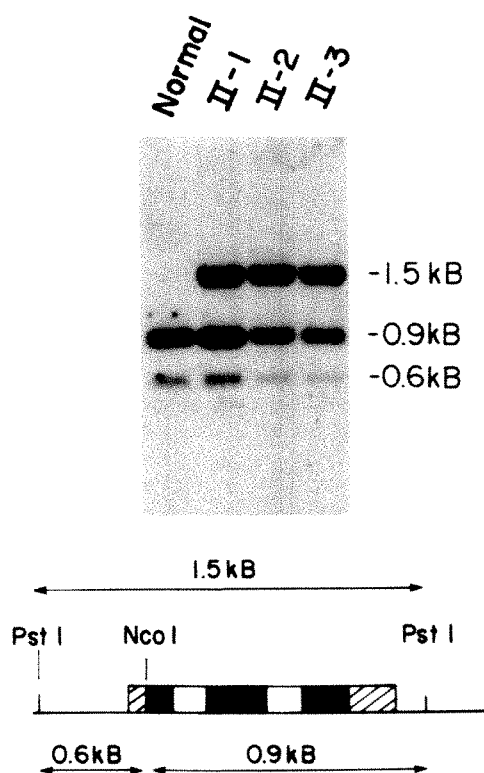


Fig 3. Direct demonstration of initiator codon mutation in family G. Genomic DNA samples were digested with *Nco*I and hybridized with the 1.5-kb *Pst*I fragment indicated in the schematic map.

an α 1-globin gene or within an α 2/ α 1 fusion gene created by a crossover 5' to the nonhomology of the normal α -globin genes in IVS-2.^{38,39}

Several molecular defects responsible for nondeletion α -thalassemia determinants have been previously described. They include a pentanucleotide deletion in the first intervening sequence of an α 2-globin gene, preventing normal RNA splicing¹⁵; chain termination mutations resulting in synthesis of highly unstable α -globin mRNA^{17,18}; a substitution in the coding region resulting in an unstable α -globin variant^{14,16,19,20}; and a substitution in the poly(A) addition signal

of the 3'-untranslated sequences of an α 2-globin gene leading to impaired polyadenylation of nascent α -globin mRNA.²¹

Initiation codon defects leading to α -thalassemia of genes have also been described. A dinucleotide deletion preceding the AUG codon was identified in an Algerian patient with HbH disease.²⁴ A single base substitution (ATG $\rightarrow$ ACG), different from that described here, was observed in the initiation codon of the α 2-globin gene in Sardinians.²⁵ This defect may account for 70% of nondeletion α -thalassemia determinants in this group.²⁶ In a single Sardinian patient, an α 1-globin gene initiation codon was inferred by Southern blot analysis.²⁶ In the family we describe, the initiation codon mutation (ATG $\rightarrow$ GTG) was present on a $-\alpha$ ^{3,7} chromosome. This mutation, like that characterized in the α 2-gene of Sardinians, generates an abnormal Southern blot pattern on *Nco*I digestion. Although nucleotide substitutions in the initiation codon leading to α -thalassemia are identifiable by enzyme digestion, more specific assays, such as those based on use of synthetic oligonucleotides, are required to assign the particular mutation.

Although the duplicated α -globin genes α 2 and α 1 encode identical α -globin polypeptides, it is striking that characterized nondeletion α -thalassemia defects, like the chain termination α -globin variants,⁴⁰ primarily affect the α 2-globin gene rather than both with equal frequency. In that α 2 mRNA is more abundant than α 1 mRNA^{18,41} in the steady state and appears to contribute to a larger fraction of α -globin synthesis,⁴² the predominance of α 2-globin gene mutations may reflect a bias in ascertainment based on differing phenotypes. Mutations in the α 1-gene, when present *in cis* to a normal α 2-gene, would be phenotypically mild. Although the mutation we have examined is contained in an α -gene with the sequence hallmarks of the α 1 type,³⁸ it is uncertain whether it arose within an α 1 gene on a normal chromosome or within an α 2/ α 1 fusion gene derived from a crossover event. Nevertheless, absence of an α 2-gene *in cis* facilitated identification of this novel α -thalassemia gene.

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Regulated Expression of Amplified Human β Globin Genes

By Deborah Rund, Carl Dobkin, and Arthur Bank

Gene therapy for the β thalassemias and sickle cell anemia will require high levels of expression of human β globin genes. One method to achieve this goal is amplification of globin genes transferred into the stem cells in the bone marrow of these patients. If the amplified genes remain normally regulated, they will then further increase their expression on being induced to differentiate along an erythroid pathway. To begin this study, we constructed a plasmid containing a neomycin resistance gene, a human β globin gene, and a wild-type DHFR cDNA, and transfected it into mouse erythroleukemia cells. All the G418-resistant clones analyzed acquired and expressed the human β

globin gene. By serial passage of the cells in increasing concentrations of methotrexate, the exogenous human β globin genes were stably amplified in all lines, and all increased their globin mRNA expression roughly proportional to their augmented copy number. Most of the clones further increased their β globin expression on addition of an erythroid stimulus (dimethylsulfoxide). These results indicate that globin gene amplification may be useful in increasing globin mRNA expression in further experiments whose goal is gene therapy.

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GENE THERAPY for disorders of hemoglobin such as the β thalassemias and sickle cell anemia will require high-level expression of human β globin genes. One approach to this goal is to amplify globin genes transferred into the bone marrow cells of these patients and then to obtain regulated expression of the amplified copies of these normal genes. The dihydrofolate reductase (DHFR) gene is amplified in the presence of the compound methotrexate (Mtx).¹⁻⁴ This is true not only for the endogenous gene but also for exogenously introduced DHFR genes that can also amplify and confer Mtx resistance with increased copy number.⁵

Variations in expression of foreign genes have been attributed to different chromosomal positions of these genes.⁶ In addition, augmentation of copy number is only variably associated with increased expression of these genes.⁷ The regulation of the amplified genes may be altered in the amplified state^{8,9}; for example, the metallothionein I gene loses its steroid regulation although its cadmium responsiveness is maintained despite increased copy number in homologous cells.⁹

When the DHFR gene is amplified, there is concomitant amplification of a large region of the genome adjacent to the DHFR gene [up to 500 kilobases (kb)].^{10,11} We used this amplification in the experiments we report to increase the human β globin gene copy number in erythroid cells and then tested the expression of these amplified genes. The results demonstrate appropriately regulated expression of amplified human β globin genes in erythroid cells. This strategy may be useful in experiments whose goal is gene therapy.

To begin these experiments, we constructed a plasmid containing a neomycin resistance (neo^R) gene,¹²⁻¹⁴ a human β globin gene with flanking sequences, and a wild-type DHFR cDNA.¹⁵ This plasmid was transfected into mouse erythroleukemia cells (MELC), and colonies resistant to G418 (a neomycin analogue) were selected and analyzed for their content of human β globin and DHFR genes.¹⁶ These clones were then subjected to Mtx selection, and changes in DNA content and RNA expression were measured.^{13,17,18} The unamplified and amplified clones were then subjected to dimethylsulfoxide (DMSO), an inducer of erythroid differentiation and hemoglobin synthesis in these cells.¹⁹ Our results indicate that the human β globin gene stably increases in copy number with Mtx selection, that expression of β globin mRNA rises concomitantly and roughly proportional to or in excess of the increase in gene content, and that

there is a further increment in expression on addition of an inducer of erythroid differentiation in most clones examined.

MATERIALS AND METHODS

Plasmid construction and transfection. The plasmid pSV2neo¹⁴ was used as the basis for this construction. A 4.4-kb *Pst*I fragment containing the entire human β^S globin gene with ~2.5 kb 5' and 0.5 kb 3' flanking DNA was ligated at the *Pst*I site of pSV2neo. A *Bam*HI/*Pvu*II fragment containing a full-length murine (0.7 kb) wild-type DHFR cDNA with an SV 40 promoter and an SV 40 polyadenylation signal was isolated from pSV2DHFR¹⁵; the ends of this fragment were treated with the Klenow fragment of DNA polymerase, and *Xho*I linkers were added. This fragment was ligated into a unique *Xmn*I site in pSV2neo to which *Xho*I linkers had also been added. The resulting plasmid, $\beta^SpSV2neo$ -DHFR, 11.8 kb in size, is shown in Fig 1.

This plasmid was transfected into MELC by protoplast fusion techniques.²⁰ After 24 hours of growth without selection, G418 (a neomycin analogue) in a concentration of 400 μ g/mL was added to the media for 72 hours, after which it was increased to 800 μ g/mL. Clones were maintained in G418 (400 μ g/mL) once selection was completed.

Cell growth, gene amplification, and induction. MELC and clones were grown in RPMI (GIBCO, Grand Island, NY) with 10% heat-inactivated (56°C) fetal bovine serum (GIBCO), 0.24% NaHCO₃, penicillin (100 U/mL), streptomycin (100 U/mL), and amphotericin B (0.25 μ g/mL) (GIBCO).

Mtx selection was performed by addition of Mtx (+ amethopterin, Sigma, St Louis) to the media for periods of ~21 days at each level of

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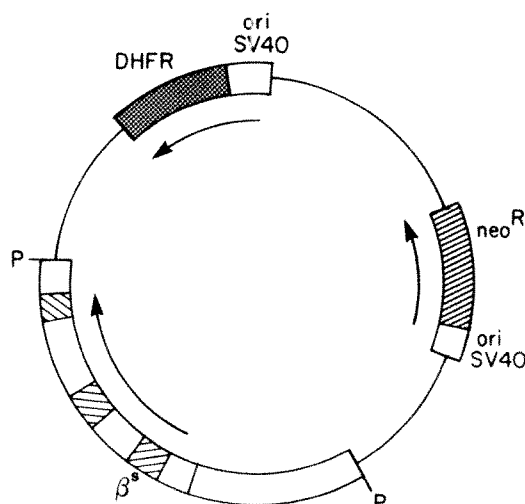


Fig 1. Diagram of plasmid β^S pSV2neo-DHFR, 11.8 kb in size. Details of construction are given in the Materials and Methods section. The plasmid contains a neomycin resistance gene under the control of an SV 40 promoter, a wild-type DHFR cDNA also under SV 40 control, and a 4.4-kb human β^S globin gene. Arrows indicate direction of transcription. P, *Pst*I restriction site.

Mtx added. Selection was begun at 2×10^{-9} mol/L Mtx, a concentration that consistently demonstrated 90% cell kill of MELC that had no exogenous DHFR genes. Cell death was maximal at day 14. Cells were then subjected to fivefold increases in Mtx concentration, ie, 10^{-8} , 5×10^{-8} mol/L, etc. In addition, MELC that had not been transfected with the plasmid were also subjected to the same increasing concentrations of Mtx. Induction of erythroid differentiation was performed on both unamplified and amplified clones by plating the cells at a density of 10^5 /mL, adding 1.6% DMSO to the media, and harvesting the cells 96 hours later.

DNA analysis. DNA and RNA were isolated from 1 to 5×10^7 cells as described.^{21,22} Southern blotting was performed by using the restriction endonucleases noted in legends to the figures according to the specifications of the manufacturer (New England Biolabs). After agarose gel electrophoresis (0.7% for DHFR gene analysis, 1% gel for β analysis), Southern blotting was performed as described.^{16,22} Three probes were used: (a) A 4.4-kb *Pst*I fragment containing the entire human β globin gene, (b) a *Hind* III/*Bgl* II fragment of pSV2DHFR containing the complete murine wild-type DHFR cDNA, and (c) an 800-bp *Pvu* II fragment of the neo^R gene. Probes were P³² labeled by nick-translation to a specific activity of $>2 \times 10^9$ counts/ μ g. The final washes were performed at $0.2 \times$ saline sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS) at 65°C.

RNA analysis. SI nuclease analysis^{12,18} was performed on MELC and clones before and after Mtx selection with and without induction. RNA was prepared as noted above under DNA analysis. Twenty-five micrograms of total cellular RNA was lyophilized and hybridized to a human β globin probe consisting of a 767-base *Eco*/Pst fragment representing the 3' end of the β gene and untranslated sequences. This probe was end-labeled using the Klenow fragment of DNA polymerase, and strand-separated on an acrylamide gel. SI nuclease analysis was performed as described.¹⁸ The appropriate protected fragment is 212 bases. In other experiments, a 565-base *Bal* I/*Bam* HI probe was used. This was inserted into the polylinker site of SP62, and labeled RNA was prepared as noted and used as probe.¹⁷ A 3' mouse β globin probe, a 324-b *Sau* IIIA/*Sau* IIIA fragment, was also used in SI nuclease experiments.

Densitometry. Densitometer tracings were performed using a Chromoscan-3 densitometer according to the manufacturer's specifications (Joyce Loebel, Malden, MA).

RESULTS

Globin gene amplification. Twenty-four clones were obtained after initial transfection with β^S pSV2neo-DHFR and G418 selection, of which six were randomly selected for analysis. On Southern blotting, all six clones acquired 6 to 26 copies of the human β globin gene as quantitated by densitometry tracings before exposure to Mtx (Fig 2). Unique junction fragments, larger in size than the plasmid, are present in all clone lanes in Fig 2 (with the exception of clone 1) and indicate integration of the plasmid into host cell DNA. Clone 1 demonstrates junction fragments on digestion with enzymes other than *Pst*I.

After exposure of each of the clones to Mtx selection at several levels, DNA was again analyzed for human β globin gene content (Fig 3). All six clones demonstrated further amplification of the transfected human β globin gene. The increase over baseline in DNA content was measured using

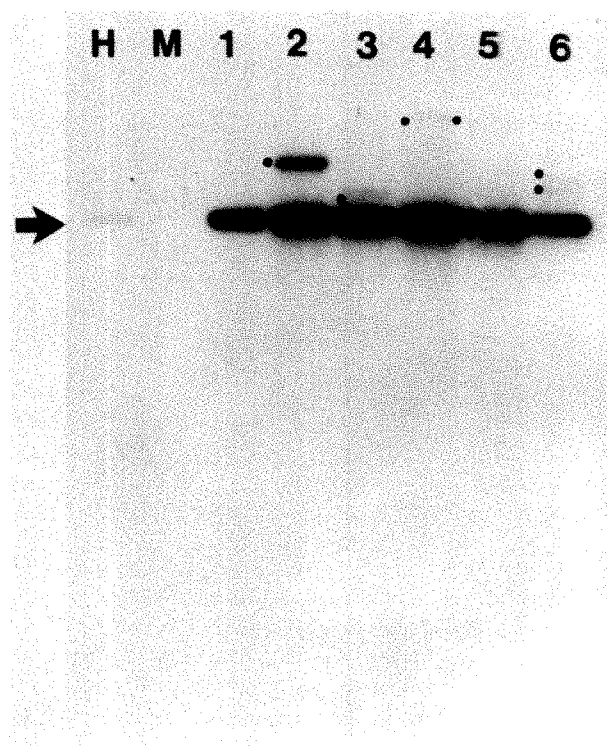


Fig 2. Human β globin gene transfer into MELC. Southern blot hybridization of DNA from six clones was performed prior to exposure to Mtx. One microgram of DNA digested with *Pst*I was used for all lanes. A human β globin gene probe was used. Lane H: Human leucocyte DNA. The signal corresponds to a diploid cell single copy. Lane M: DNA from MELC without plasmid. There is no cross-hybridization signal from mouse endogenous globin sequences. Lanes 1 through 6: Six clones transfected with plasmid; 6 to 26 copies of the human gene have integrated. Arrow (left) indicates appropriate 4.4-kb *Pst*I fragment. The junction fragments apparent in each clone are indicated by dots to the left of the fragment.

Fig 3. Amplification of human β globin genes in MELC. Southern blot hybridization of DNA from six clones before and after Mtx selection. All lanes were digested with *Pst*I. Arrow (left) indicates the appropriately sized 4.4-kb *Pst*I fragment. Exposure time at -70°C was 16 hours. Lane M: DNA (5 μg) from MELC without plasmid. Lane P: Plasmid DNA (50 pg). All remaining lanes contain 1 μg cellular DNA from six clones (A through F). A1, B1, C1, D1, E1, and F1: Clones before Mtx selection. A2, B2, and C2: Clones after exposure to 10^{-8} mol/L Mtx. A3, B3, C3, D2, E2, and F2: Clones after exposure to 5×10^{-8} mol/L Mtx.

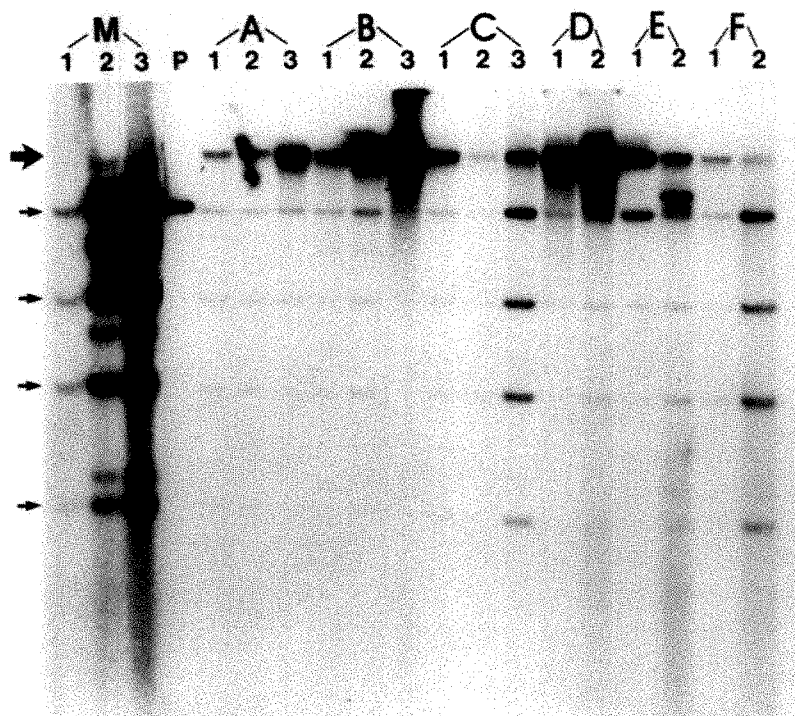
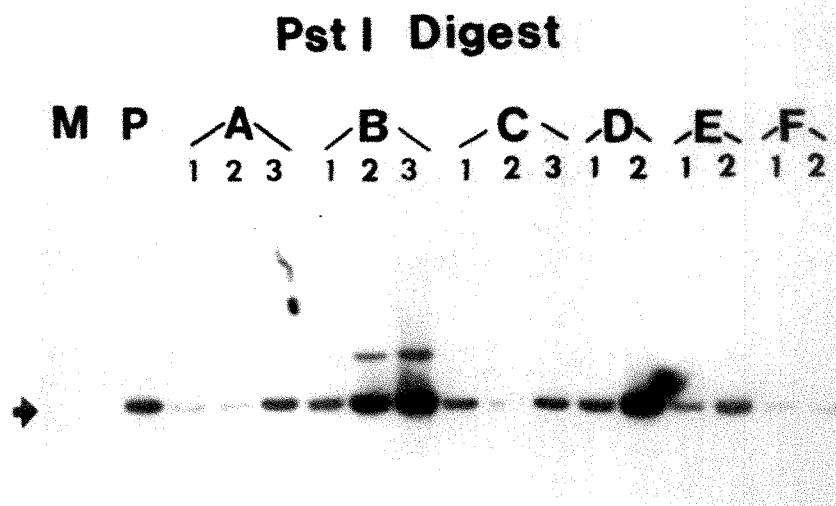


Fig 4. Amplification of DHFR genes in MELC. Southern blot analysis of MELC before and after Mtx selection. DNA was digested with *Xba*I except for lane P, which was digested with *Sma*I. *Xba*I does not cut the plasmid but does cut within endogenous DHFR sequences. The probe used was a *Hind*III/*Bgl*II fragment of pSV2DHFR containing the complete murine DHFR cDNA. Exposure time at -70°C was 96 hours. Lanes M1 through M3: DNA (5 μg) from MELC without plasmid before (M1) and after (M2, M3) exposure to Mtx at concentrations of 10^{-8} and 5×10^{-8} mol/L Mtx, respectively. There is amplification of the endogenous DHFR bands indicated by the four small arrows (left). Lane P: Linearized plasmid DNA (50 pg). All lanes A through F contain 1 μg cellular DNA from the six clones shown in Fig 3 before and after Mtx selection. The large (and highest) arrow at left indicates the position of high-mol-wt integrated exogenous DHFR genes present in all six clones and absent in the control (M). Lanes A1, B1, C1, D1, E1, and F1 are the clones before Mtx selection. Lanes A2, B2, and C2 are the clones following exposure to 10^{-8} mol/L Mtx. Lanes A3, B3, C3, D2, E2, and F2 show the same clones after exposure to 5×10^{-8} mol/L Mtx. There is clear amplification of the exogenous DHFR genes in clones A, B, and D. In these clones and clone E, the endogenous DHFR gene bands serve as controls for the constant amount of DNA added to each lane. In clones C and E, there is amplification of the endogenous DHFR genes at the higher Mtx levels shown.

densitometry (Fig 3). Clone A demonstrates no significant amplification on exposure to the lower level of Mtx, a 2.5-fold increase on exposure to 5×10^{-8} mol/L Mtx, and a 10-fold increase at 2.5×10^{-7} mol/L Mtx. Clone B amplifies 2.1-fold over baseline at 10^{-8} mol/L and 3.3-fold at 5×10^{-8} mol/L Mtx. Clone C shows no increase at the Mtx levels shown, but amplifies 8-fold at 2.5×10^{-7} mol/L Mtx. Clone D shows a 2.5-fold increase, and clone E shows a smaller increase, at 5×10^{-8} mol/L Mtx. Clone F does not amplify at the Mtx concentration shown here (5×10^{-8} mol/L), but amplifies 8-fold on exposure to 2.5×10^{-7} mol/L Mtx. Thus, all six clones amplify their β globin gene content in response to Mtx selection—one at 10^{-8} mol/L, three at 5×10^{-8} mol/L, and two at 2.5×10^{-7} mol/L Mtx. This clonal variation in propensity to amplification of transfected genes has been noted by other researchers.⁶ On removal of Mtx from the media for ~100 generations of cell passage, the amplified human β globin gene copy number was unchanged, indicating stable amplification (data not shown).

DHFR gene amplification. The clones were also examined for their DHFR gene content. MELC not transfected with the plasmid but exposed to the same amounts of Mtx were used as the control cells. Cellular DNA was digested with *Xba*I; this enzyme does not cut within the transfected plasmid sequences but does cut within the endogenous mouse DHFR gene. Thus, a band of larger size than that of the plasmid is expected if the plasmid has been integrated into cellular DNA; this is the result shown in Fig 4. All clones contain bands of mol wt larger than the linear plasmid, indicating integration of the exogenous gene into host sequences. Clones A, B, and D show a clear and progressive

increase in only the exogenous DHFR gene, with increasing amounts of Mtx. These clones showed 5- to 20-fold amplification of the exogenous DHFR genes (Fig 4). Clone C shows no increase in the exogenous gene at the higher Mtx level (the apparent decrease at the lower level of Mtx is due to less DNA in this lane) and the endogenous DHFR gene (lower bands) clearly amplifies at the higher Mtx dose (Fig 4). Clone C shows amplification of the exogenous DHFR gene at 2.5×10^{-7} mol/L, the dose that also shows β globin gene amplification. Clone E is uninterpretable at the Mtx level shown due to multiple exogenous bands, but clearly amplifies only the exogenous gene at 2.5×10^{-7} mol/L Mtx (data not shown). In no case was the endogenous human β globin amplified at a level at which DHFR did not amplify, nor did the reverse occur. The fold increases of amplification of the β globin gene and the DHFR gene as measured by densitometry are nearly identical, indicating that they are part of the same amplification unit. The apparent differences in intensity of the signals in Figs 3 and 4 are due to differences in exposure time with the DHFR blot (Fig 4) exposed four times longer to allow for visualization of the faint endogenous DHFR bands.

The endogenous DHFR gene bands in clones A, B, D, and E in Fig 4 provide a control for the constant amount of DNA used in these experiments, and the specific amplification of only those genes linked to the exogenous DHFR gene in these lines. Southern blotting performed with a *neo*^R probe demonstrates amplification of the *neo*^R gene in all clones, at levels consistent with their degree of amplification of DHFR and human β ^S (data not shown). No amplification of endogenous mouse β globin sequences is seen (data not shown) again

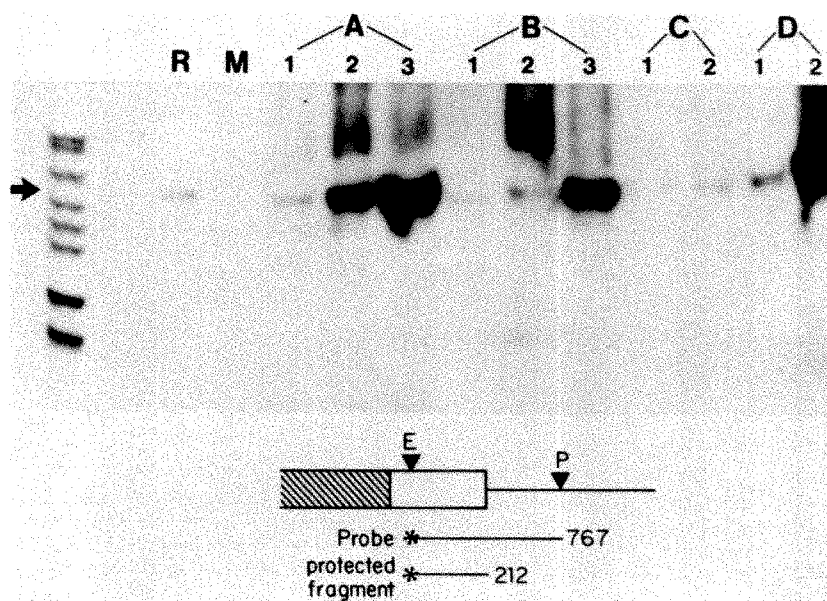


Fig 5. Human β globin mRNA expression in MELC. S1 nuclease analysis of RNA was performed on MELC before and after Mtx selection. A 767-base *Eco*-*Pst* fragment representing the 3' end of the β globin gene was used as probe. The appropriate protected fragment is 212 bases (arrow left). The marker lane is an *Hpa*II digest of pBR. Lane R: Human reticulocyte RNA (25 ng). All remaining lanes contain 25 μ g total cellular RNA. Lane M: RNA from MELC without plasmid. Lanes A through D: RNA from four separate clones before (A1, B1, C1, D1) and after exposure to Mtx selection. Lanes A2, B2, and C2: Following exposure to 10^{-8} mol/L Mtx. Lanes A3, B3, and D2: Following exposure to 5×10^{-8} mol/L Mtx. There is a progressive increase in human β globin mRNA in cells that have been exposed to Mtx. Clones are labeled as in Fig 3 except that A and B have been reversed.

confirming the specific amplification of the exogenous DHFR-linked genes.

RNA analysis of clones. The expression of the exogenous human β globin gene was examined using a human β 3' probe that does not cross-hybridize to mouse endogenous globin RNA (Fig 5). All clones express the β globin gene; there is clonal variability in the level of expression prior to amplification. Furthermore, all clones increase their expression of human β globin mRNA with exposure to increasing amounts of Mtx. The increase in expression of the transferred genes is proportional to the increase in copy number, but greater in actual quantity than the increase in their DNA content. The fold increase of expression over baseline of the six clones is 3.3- to 20-fold (Fig 5) as compared with the 2.1- to 8.0-fold increase in DNA content at an Mtx level of 5×10^{-8} mol/L. In every instance, the relative increase in expression is greater than the increase in gene content (Fig 5

as compared with Fig 3). This propensity to overexpression in amplified DHFR genes has been noted by other researchers¹ as well as in genes coamplified with an exogenous DHFR gene.²³

Analysis using another radiolabeled probe (a *BalI/BamHI* fragment that spanned the 5' end of the human β globin mRNA) showed that all the transcripts were appropriately initiated and that IVS 1 was appropriately spliced out both before and after amplification (data not shown). Thus, there is an increase in intact human β globin mRNA expression in erythroid cells containing increased copies of the human β globin gene by amplification.

Regulated β globin mRNA expression. To address the question of whether the amplified β globin genes are expressed in a properly regulated manner in these erythroid cells, MELC were exposed to DMSO, a compound known to increase erythroid differentiation and hemoglobin synthesis

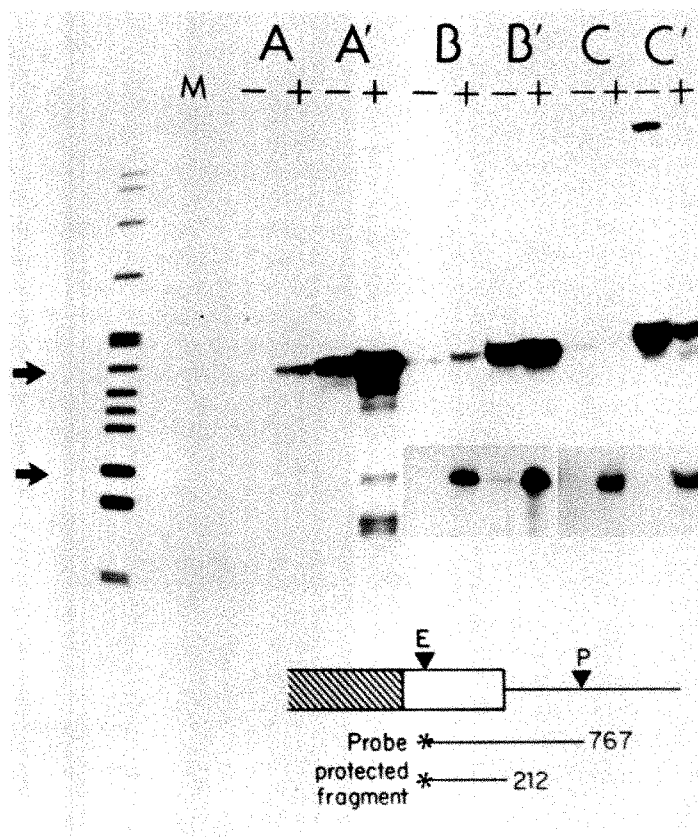


Fig 6. Induction of human β globin mRNA expression in MELC after amplification. S1 nuclease analysis of clones before and after Mtx selection. All lanes contain 25 μ g total cellular RNA. The probe is as described for Fig 5. Lanes A, D, and C: Three clones prior to Mtx selection. Lanes A', D', and C': The same three clones after amplification by Mtx selection. Both unamplified and amplified cells were harvested before [(uninduced) designated as minus (-)], or after 96 hours of exposure to 1.6% DMSO [(induced) designated as plus (+)]. Arrow (left) indicates the appropriately protected 212-base fragment. The marker lane at left is an *HpaII* digest of pBR. Lane M: RNA from MELC without plasmid. Lane A: Unamplified clone, uninduced (A-) and induced (A+). Signal is increased following induction. Lane A': Amplified (A'+) clones show an increase in signal after DMSO exposure as compared with (A'-). Clone B also shows an increase in globin mRNA expression in both the unamplified and amplified clones following induction. Clone C: No induction of unamplified or amplified clones. The apparent decrease in lane (C'+) is due to the presence of less RNA in this lane, and multiple subsequent analyses showed no change in expression of this clone with induction in either the unamplified state or after Mtx selection. Three other clones are not shown, two of which show inducible expression of the amplified β globin gene, and one of which does not. The level of Mtx exposure in all these clones is 5×10^{-8} mol/L Mtx. Inset (lower right) under D,D' and C,C' is an S1 nuclease analysis of these RNAs using a mouse 3' β probe (a 324-base *SauIIA/SauIIA* fragment) of comparable specific activity to that of the human β probe, but exposed for only 8 hours instead of 16.

in these cells.¹⁹ In five of six clones examined in the unamplified state, there is a significant increase in expression of human β globin mRNA with DMSO as compared with that seen in its absence, as reported by other researchers.^{24,25} In four of these clones, there is also an increase in the expression of the amplified human β globin genes in the presence of DMSO (Fig 6). One of the six clones is uninducible both when unamplified and amplified, and one is inducible only when unamplified. Densitometry scanning quantifies these fold increases as 1.4- to 2.5-fold induction. Clones have similar fold increases with induction of both their unamplified and amplified counterparts. These data indicate that appropriate regulation of transfected amplified genes occurs at most of the chromosomal positions into which these genes are integrated. This also suggests that all the *cis*-acting sequences required for tissue-specific appropriate expression of these genes are present in the amplified genes at these locations, and can respond to the *trans*-acting factors in the erythroid cell's environment to increase human β globin production.

All clones, including the clone that is not inducible for human β globin, were inducible for expression of mouse endogenous globin mRNA using a 3' probe with no cross-hybridization to human β globin mRNA (Fig 6 and data not shown). This suggests that the defect in globin gene regulation when present is specific for the exogenous gene and related to certain chromosomal positions.

The level of expression of the transfected human β globin genes was assessed by comparing it with the expression of induced endogenous mouse β globin genes using radioactive probes of comparable lengths and comparable specific activities. Although there is considerable variation in the level of expression of the exogenous gene in different clones, the level of induced expression of human β globin mRNA in the clones after amplification at an Mtx level of 5×10^{-8} mol/L is 33% to 100% that of the mouse endogenous β genes.

DISCUSSION

In these experiments, transfer, amplification, and appropriate expression of human β globin genes at foreign chromosomal positions in mouse erythroid cells were demonstrated. The final level of expression of these amplified genes is roughly comparable to the expression of the endogenous mouse β globin genes. Thus, it is conceivable that this

strategy applied to human bone marrow cells might be useful in gene therapy of disorders of human hemoglobin.

There is preferential amplification of the exogenous wild-type DHFR cDNA over the endogenous DHFR gene. We believe this is due to several circumstances. First, G418 selection ensures the presence of an exogenous DHFR gene in the clones examined. Because the rate of transfection of exogenous genes is so low ($1/10^5$ to 10^6), this selection is required for the isolated clones to contain the exogenous gene. The preferential amplification of the exogenous DHFR cDNA in most of the clones may be due solely to its strong SV 40 promoter as compared with a weaker endogenous DHFR promoter. Alternatively, G418 may select for a chromosomal environment that permits expression of the neo^R gene and may simultaneously favor expression of the transferred DHFR cDNA present on the same plasmid at the same location. This favored chromosomal environment may also explain the high level of expression of human β globin genes before induction and partially explain the lesser fold increase in expression of the exogenous β gene after induction.

To provide efficient gene transfer into the bone marrow of animals, it will be necessary to use retroviral vectors.²⁶⁻³⁰ Recent experiments have demonstrated that ecotropic and amphotropic helper viruses can be used to infect mouse and monkey bone marrow cells and human bone marrow cells in culture.²⁶⁻³⁰ In these experiments, prior selection with G418 is used to select those cells that have integrated a retroviral vector expressing a neo^R gene. Ablated bone marrow is totally repopulated with cells containing the neo^R gene.²⁶⁻²⁸ This is precisely the same strategy that could be used with the construct described here; selection with G418 would ensure that DHFR amplification occurs exclusively from the exogenous and not the endogenous DHFR genes in the same manner as demonstrated in MELC in these experiments. This in turn would strongly favor human β globin gene amplification. Thus, a retroviral vector containing neo^R, DHFR, and human β globin genes infected and expressed in human bone marrow cells may be especially useful in experiments whose goal is the correction of human β globin synthesis in β thalassemia and sickle cell anemia.

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Abnormal Stimulated Adherence of Neonatal Granulocytes: Impaired Induction of Surface MAC-1 by Chemotactic Factors or Secretagogues

By Donald C. Anderson, Katherine L. Becker Freeman, Barbara Heerdt, Bonnie J. Hughes, Richard M. Jack, and C. Wayne Smith

To identify possible secretory determinants of impaired hyperadherence and stimulated migration of neonatal granulocytes (NGs), we performed correlative studies of: (a) specific granule content and exocytosis, (b) secretagogue-mediated upregulation of f-met-leu-phe (fMLP) receptors, (c) the chemotactic induction of the adhesive glycoproteins Mac-1 α (complement receptor 3) and β , and (d) morphometric assessments of specific (peroxidase negative) granule depletion following chemotactic stimulation. Lactoferrin (LF) content of NG suspensions (cord blood or peripheral blood cells) was profoundly diminished (mean $\pm$ SD 51% $\pm$ 18% of normal) as compared with healthy adult granulocytes (AGs). Despite diminished cellular content, LF release by NG suspensions in response to fMLP was comparable to that of AGs. In contrast, LF release by NG suspensions was significantly diminished in response to phorbol myristate acetate (PMA) or calcium ionophor A23187 and/or during stimulated cell spreading, experimental conditions promoting overall greater LF depletion than chemotactic stimuli. In addition, NGs demonstrated an impaired capacity to upregulate fMLP receptors in response to PMA or A23187 when tested under the same experimental conditions. Baseline expression of the adhesive glycoproteins Mac-1 α and β on NG surfaces was normal, but induction or upregulation of these proteins by

chemotactic concentrations of fMLP, C5a as well as secretory (high) concentrations of PMA and A23187, was significantly diminished as compared with AGs. In contrast, chemotactic induction of the surface expression of the complement receptor-1 (CR-1) on NGs was normal. An impaired induction of Mac-1 α or β was directly related to an impaired enhancement of adherence of NG in response to fMLP over a chemotactically relevant concentration range (10^{-10} to 10^{-7} mol/L). Moreover, in blocking-incubation experiments using anti-Mac-1 α/β monoclonal antibodies (MAbs), significantly less inhibition of adherence by these MAbs was evident with fMLP-stimulated NG as compared with AG suspensions. Under selected chemotactic conditions, ultrastructural assessments of NGs demonstrated diminished peroxidase-negative granule loss in association with diminished granule-membrane fusion and the "addition" of plasma membrane. These studies suggest that abnormal expression of multiple surface determinants derived from peroxidase-negative granules or other intracellular pools may contribute to deficient chemotaxis or other inflammatory functions of NGs. Specifically, they indicate that diminished stimulated adherence of NGs is functionally linked to an insufficient chemotactic induction of surface Mac-1 protein subunits.

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PREVIOUS STUDIES in human neonates and neonatal animal models have consistently documented abnormalities of neutrophil mobility that are believed to contribute to the neonate's infectious susceptibility and morbidity.¹⁻⁸ We previously studied and documented abnormalities in one important determinant of leukocyte mobility, the adherence properties of neonatal granulocytes (NGs) under conditions of chemotactic stimulation.¹ Although molecular events con-

tributing to cellular adherence are incompletely understood, observations in vivo have revealed that granulocytes adhere preferentially to vascular endothelium adjacent to a site of inflammation before their migration into tissues.⁹ These events are facilitated by the occupancy of membrane receptors by C5a and other byproducts of inflammation which elicit a sequence of incompletely understood mechanisms that enhance cellular adherence properties.¹⁰⁻¹⁵

Complex secretory contributions to stimulated granulocyte motility and adherence have been suggested by several lines of investigation.¹³⁻²⁴ The process of extracellular release or exocytosis of specific granules or other secretory vesicles represents a highly integrated mechanism by which cell surface adherence properties are altered in response to inflammatory stimuli. The perigranular membrane of specific granules provides a source of "new membrane" required for morphologic alterations of motile or hyperadherent cells.¹⁵ Some experimental evidence suggests that specific granules also represent an intracellular pool from which receptors for the chemotactic factor fMLP^{18,19} and the adhesive glycoproteins Mac-1 (iC3b receptor) and p150,95^{12-14,23-25} are up-regulated to cell surfaces by chemotactic or secretory stimuli. In addition, the extracellular release of specific granule constituents, including lactoferrin (LF) or other cationic proteins, modulates cell surface charge or other biophysical properties that contribute to cellular adherence.^{21,22} The physiologic and potential pathologic contributions of specific granule-dependent functions of neutrophils have been emphasized by observations and studies of patients with genetic deficiency of neutrophil-specific granules^{20,21} and of individuals with a genetic deficiency of the leukocyte

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Mac-1, LFA-1 glycoprotein family.^{12-14,24} Infectious susceptibility and impaired inflammatory functions recognized in both pathologic entities have been partially attributed to abnormalities of neutrophil and/or monocyte adherence. Of considerable relevance to our studies reported herein, Ambruso and co-workers recently reported diminished LF content as well as quantitative and qualitative abnormalities of specific granules in NGs.²⁶ The relationship(s) of their findings to impaired chemotaxis or other adherence-dependent functions of NGs have not been previously evaluated.

To identify possible secretory determinants of impaired hyperadherence and stimulated migration of NGs, we performed correlative studies of: (a) specific granule content and exocytosis; (b) secretagogue-mediated up-regulation of fMLP receptors; (c) the induction of the "adhesive" glycoproteins Mac-1 α , β , and p150,95 α by chemotactic or secretory stimuli; and (d) ultrastructural and morphometric assessments of peroxidase-negative granule depletion or "membrane addition" effected by chemotactic stimuli. Our studies suggest that abnormal expression of multiple surface determinants derived from specific granules or other intracellular pools may contribute to deficient chemotaxis or other inflammatory functions of NGs. In addition, our findings indicate that impaired chemotactic induction of Mac-1 subunits represents a major pathogenic mechanism accounting for their diminished stimulated adherence properties. Finally, these studies provide further evidence for diverse secretory contributions to neutrophil inflammatory functions.

MATERIALS AND METHODS

Blood samples. Heparinized venous blood was drawn from the placentas or peripheral veins of normal full-term or premature infants within 5 minutes of birth. Peripheral blood samples were also drawn from healthy neonates (age 1 to 30 days), healthy maternal pairs, Mac-1, LFA-1-deficient individuals, and healthy adult laboratory donors. All blood samples were processed within 10 minutes after phlebotomy. Informed consent was obtained from patients or their legal guardians prior to obtaining blood samples.

Isolation of granulocytes. Granulocytes were purified from heparinized, dextran-sedimented venous blood samples over Ficoll-Hypaque gradients and suspended in Dulbecco's phosphate-buffered saline (DPBS) (GIBCO Laboratories, Grand Island, NY), pH 7.4, containing 0.2% dextrose as described.¹ For use in *N*-formyl-methionyl-leucyl-[³H]-phenylalanine (fML[³H]P) binding studies, erythrocytes were eliminated by hypotonic lysis.¹ To minimize degranulation or "spontaneous up-regulation" of C3b receptors (CR-1) or Mac-1 glycoprotein (iC3b receptor or CR-3) surface expression during cell purification, anticoagulated blood samples were processed immediately at 4°C, and all purification procedures were performed at 0°C to 4°C with ice-cold buffers as described by Fearon and Collins.²⁷

Preparation of reagents. Stock solutions of f-Met-Leu-Phe (fMLP) (Sigma Chemical, St Louis, MO) and fML[³H]P (New England Nuclear, Boston) were prepared in DPBS. A low-mol-wt chemotactic factor (CF) referred to as C5a in this article, was prepared from activated human sera.²⁸ Zymosan, calcium ionophore A23187, phorbol myristate acetate (PMA), phenylmethyl sulfonyl fluoride (PMSF), 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma), FITC-goat anti-mouse IgG (Boehringer Mannheim, Indianapolis), and FITC-goat anti-rabbit IgG (ICS Immunobiologicals,

Naperville, IL) were obtained commercially. Stock solutions of LF purified from human milk (Sigma) were prepared in barbital buffer as previously described.²⁹ The IgG fraction of a goat anti-human LF antiserum was obtained from Atlantic Antibodies, Scarborough, ME.

Monoclonal antibodies. MAbs directed against the α subunits of Mac-1 (α M), LFA-1 (α L), and p150,95(α X) and their common β subunits were prepared as described.³⁰ Anti- α M MAbs included OKM-1 and LM 2.1.³¹ The anti- α LMAb TS1/22,³⁰ the anti- α X MAb SHCL-3³² and the anti- β MAb TS1/18,³⁰ and the P3X63K nonimmune ascites³⁰ were also used. Control preparations included a F(ab')₂ fragment of rabbit IgG directed against the human CR-1 (provided by Dr P.S. Changelian, Harvard Medical School),²⁷ an anti-CR1 MAb ("YZ-1"),³³ and a MAb directed at HLA A₁B₁ (W6/32).³⁴ IgG1 from TS1/18 ascites fluid, and IgG2b from LM 2/1 culture supernatants were obtained by passage over Protein A-Sepharose (Pharmacia, Piscataway, NJ) columns as described for mouse immunoglobulins by Ey et al.³⁵ TS1/18 and LM 2/1 F(ab')₂ were prepared by digestion with cysteine-activated papain, as described by Parham.^{31,36}

Immunofluorescence flow cytometry. Indirect immunofluorescence assessments of intact granulocytes were performed by using saturating concentrations of MAbs and FITC-conjugated goat anti-mouse IgG or goat anti-rabbit IgG F(ab')₂. Surface-stained cells were fixed in 1% paraformaldehyde and analyzed in a flow cytometer (Coulter Epics V; Coulter Electronics, Hialeah, FL) as previously described.¹³

Assay of granule-associated enzymes. Unstimulated or stimulated granulocyte suspensions (5×10^6 cells/mL DPBS) were immersed in an ice bath to terminate extracellular release conditions and centrifuged at 800 g for 5 minutes. Supernatants were carefully aspirated, and cell pellets were suspended in 0.5 mL DPBS containing 1.0 mL of Triton X-100 surfactant/L and then sonicated on ice for 120 seconds. Cell sonicates were diluted 50-fold with 2 mmol/L PMSF, and both supernatants and sonicates were stored at -70°C prior to enzyme analysis.

A radiometric technique previously described was used to assay for LF.²⁹ In brief, purified anti-LF antibody was radioiodinated using lactoperoxidase immobilized on Sepharose beads. Polystyrene beads (6-mm diameter; Clifton Plastic, Clifton Heights, PA) were coated with anti-LF IgG (200 $\mu\text{g/mL}$) in freshly prepared carbonate buffer (0.2 mol/L, pH 9.4) by overnight incubation at 4°C. Unsaturated bead binding sites were quenched in carbonate buffer containing fetal calf serum (FCS) and Tween 20 polyoxyethylene sorbitan monolaurate. To each tube containing a single antibody-coated bead, 50 μL sample (purified LF, cell sonicates, or their supernatants) and 200 μL diluent (100 $\mu\text{L/L}$ of FCS, in DPBS) were gently mixed for 1 hour at 21°C. Washed beads were incubated with radiolabeled antibody (diluted 500-fold in FCS-DPBS) for 1 hour prior to additional washing. Nonspecific binding values determined by substituting diluent for test samples were subtracted from all results. Nonspecific binding constituted <1% of total counts. As described for lactoferrin analysis, cell-free supernatants or sonicated cell preparations were also assayed for lysozyme,¹² β glucuronidase,¹² or vitamin B-12 transport protein.³⁷

fML[³H]P binding assay. Modifications of methods previously described were used.^{1,19} Triplicate suspensions of unstimulated or prestimulated granulocytes (5×10^6) were incubated in 100 μL DPBS containing 40 nmol/L fML[³H]P or 40 nmol/L fML[³H]P plus 40 $\mu\text{mol/L}$ unlabeled fMLP at 0°C with gentle mixing. Reactions were terminated after 12 min. of incubation by rapid dilution with 2 mL ice-cold DPBS followed by rapid filtration and washing (5 mL twice) of mixtures through Whatman GFC glass fiber filters (Whatman, Clifton, NJ).¹ Washed filters were placed into 10 mL Scintia Verse II scintillation cocktail, and radioactivity was quanti-

Table 1. Primary and Secondary Granule Content of NGs, Healthy AGs or Mac-1, LFA-1-Deficient Granulocytes

Patient Group	(n)	β -Glucuronidase (nmol/10 ⁷ cells)	Lysozyme (μ g/10 ⁷ cells)	LF (μ g/10 ⁷ cells)	Vitamin B12 Transport Protein (ng/10 ⁸ cells)
Neonates	(34)	308 $\pm$ 12*	7.88 $\pm$ 1.9	30.6 $\pm$ 6.1†	6.7 $\pm$ 2.4‡
Healthy adults	(32)	320 $\pm$ 18	8.32 $\pm$ 1.8	59.6 $\pm$ 5.5	8.8 $\pm$ 0.5
Mac-1, LFA-1 deficient	(3)	290 $\pm$ 18	8.51 $\pm$ 2.1	54.1 $\pm$ 3.9	8.1 $\pm$ 2.1

*Mean $\pm$ SD.† $P < .001$ as compared with healthy adult or Mac-1, LFA-1-deficient groups.‡ $P < .05$ as compared with healthy adult or Mac-1, LFA-1-deficient groups.

tated in a Packard TriCarb liquid scintillation spectrometer. Non-specific binding, defined as the amount of binding occurring in the presence of a 1,000-fold excess concentration of fMLP, represented 20% to 30% of total binding values. Specific binding was defined as the total amount of fMLP[³H]P bound minus that proportion nonspecifically bound.

Ultrastructural evaluations: Granule characterization and quantitation and morphometric assessments. The experimental conditions for ultrastructural evaluations were based on preliminary experiments to determine optimal stimulation conditions in shape change assays. As shown by Nomarski optics, stimulation with fMLP (1 or 100 nmol/L) for 20 seconds elicited cell activation evidenced by circumferential membrane ruffling but no bipolar shape change. For transmission electronmicroscopy preparation, stimulated cell suspensions were fixed in 1.8% (vol/vol) glutaraldehyde in 0.2 mol/L sodium cacodylate made in 1% sucrose, for 1 hour at 37 °C followed by up to 96 hours at 4 °C. After being washed in 0.01 mol/L sodium cacodylate, with 7% (vol/vol) sucrose, pH 7.4, cells were resuspended in DAB 0.5 mg/mL and 0.05 mol/L Tris-HCl, pH 7.6, made 0.01% (vol/vol) hydrogen peroxide and 1% (wt/vol) sucrose. Following 30-minute incubation at room temperature, neutrophils were washed three times with 0.05 mol/L acetate-veronal buffer (A-V buffer), pH 7.4, and postfixed with 1% (wt/vol) osmium tetroxide in A-V buffer containing 5% sucrose for 1 hour. Cells were washed twice with A-V buffer and stained en bloc for 1 hour with 0.5% (wt/vol) uranyl acetate dissolved in A-V buffer containing 4% sucrose. Gold-silver sections were stained with saturated aqueous uranyl acetate and counterstained with Reynold's lead citrate.

Sections were examined using a JEOL 100S or 100 CX transmission electron microscope at 60 kV. To standardize the ultrastructural granule quantitations and morphometric measurements, negatives (7,000 $\times$) were taken only if the cell profile contained at least three nuclear lobes and/or the centriole.

Intracellular granule characterizations were made based on cytochemical staining. Primary granules (evidenced by the DAB reaction) were easily distinguishable from the secondary granules. Intracellular granule quantitation assessments were expressed as the average number of peroxidase-positive or peroxidase-negative granules per neutrophil profile. Measurements of cell perimeter and area were made on a Houston graphics tablet interfaced to an Apple Computer equipped with software by Optomax. Ratios of surface to volume (S/V) were calculated from area and perimeter measurements of micrographs based on the formula: $S/V = 3/2 (P/A)$, where P = perimeter and A = area. Surface membrane area was calculated from the S/V using 397 μm^3 as the mean cell volume as previously reported.¹⁵

Adherence assays. Granulocyte adherence to serum-coated glass substrates was evaluated as previously described using Smith-Hollers chambers.¹⁰ The capacity of granulocytes to anchor (spread) on glass coverslips placed in 35-mm diameter plastic Petri dishes was assayed as described.³⁸ Supernatants of these preparations were assayed for granule-associated enzymes as described above.

Statistical analysis. Student's t test was used to determine statistical significance between the means of control and stimulated values among both neonatal and healthy adult individuals in most assays. Unless otherwise stated, all P values refer to the two-tail test. For comparisons of calculated surface area between neonatal and adult neutrophil profiles, Bonferroni T tests were applied. Linear regression analysis and statistical programs to generate an exact 95% confidence interval around regression lines were used to analyze correlations of adherence increments v surface protein expression values.

RESULTS

Quantitation of granule-associated proteins. The granulocyte content of granule-associated proteins of healthy full-term neonates ($n = 31$), premature neonates ($n = 3$), healthy adults ($n = 32$), or Mac-1, LFA-1-deficient patients ($n = 3$) is shown in Table 1. Mean values for LF and vitamin B-12 transport protein content among neonatal suspensions were significantly diminished (LF; $P < .001$, vitamin B-12 transport protein; $P < .05$ as compared with healthy adult or Mac-1 deficiency mean values), whereas mean values for β -glucuronidase and lysozyme determinations were comparable among all test groups. Among neonates tested, the lowest LF values were observed in those of three premature infants. LF content of cord blood granulocytes ($n = 20$ samples) was comparable to that of cells obtained by phlebotomy from neonates aged 1 to 30 days ($n = 14$) and was unrelated to the mode of delivery ($P = .5$, vaginal v cesarean section delivery).

Table 2. LF Release by NGs or Healthy AGs in Response to f-Met-Leu-Phe

Experimental Conditions*	Neonates†	Healthy Adults‡
DPBS	0.72 $\pm$ 0.31 (2.4)§	0.84 $\pm$ 0.1 (1.4)
f-Met-Leu-Phe		
0.1 nmol/L	0.84 $\pm$ 0.41 (2.8)	0.91 $\pm$ 0.21 (1.5)
1.0 nmol/L	1.35 $\pm$ 0.60 (4.5)	1.22 $\pm$ 0.31 (2.1)
10.0 nmol/L	1.86 $\pm$ 0.80 (6.0)	2.17 $\pm$ 0.61 (3.5)
100.0 nmol/L	2.74 $\pm$ 1.10 (9.1)	3.21 $\pm$ 0.74 (4.7)

*Cell suspensions were prewarmed for 5 minutes, at 37 °C and then incubated at 37 °C for 20 minutes with or without f-met-leu-phe, after which reaction tubes were immersed in an ice bath to terminate release conditions.

†Pooled data from analyses of four cord blood samples and four specimens obtained by phlebotomy from neonates aged 2 to 16 days.

‡Pooled data from analyses of six healthy adult laboratory donors.

§LF release expressed as $\mu\text{g}/10^7$ granulocytes (mean $\pm$ 1 SD) and, in parentheses, mean percentage of release of total cell content. (Neonates averaged 30 $\mu\text{g}/10^7$ cells. Adults averaged 60 $\mu\text{g}/10^7$ cells.)

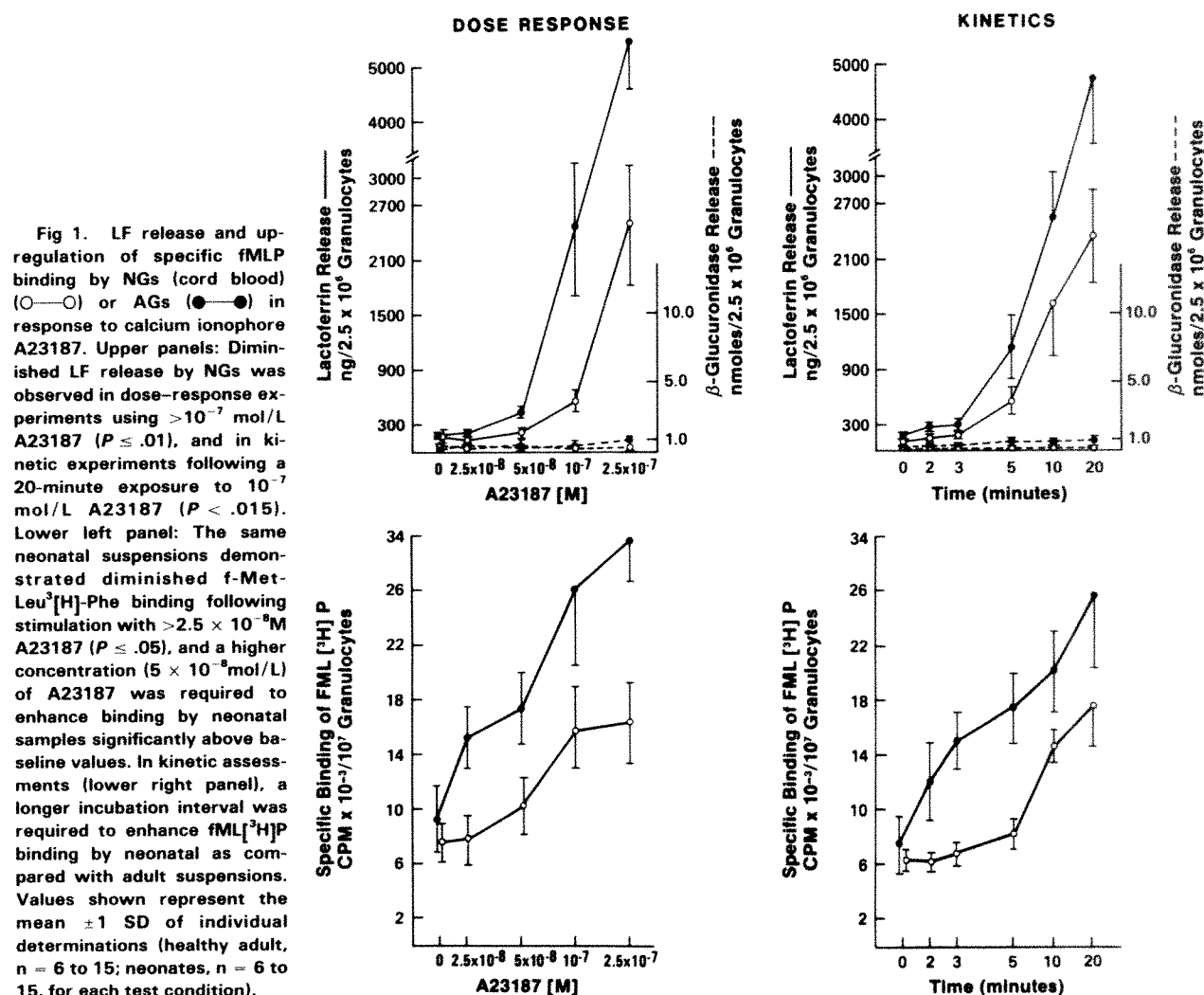
LF release. The release of LF by suspensions of NGs or healthy adult granulocytes (AGs) in response to fMLP stimulation is summarized in Table 2. Following a 20-minute exposure at 37 °C to 0.1, 1, 10, or 100 nmol/L fMLP, neonatal suspensions demonstrated extracellular LF release equivalent to that of healthy adult suspensions. As a result of their diminished total LF content, calculated percentage release values were significantly ($P \leq .02$) greater for neonatal suspensions under each test condition. In contrast, abnormalities of LF release by suspensions of NGs were observed in response to the secretagogues A23187 and PMA under conditions eliciting overall greater release than occurred following fMLP stimuli. Stimulating conditions chosen were shown to elicit preferential release of specific granules; essentially no release of β glucuronidase was observed under each of the incubation conditions illustrated in Figs 1 and 2. Assessments of fMLP receptor up-regulation were concurrently performed on the same test suspensions in response to these secretory stimuli (as discussed below).

Following a 20-minute exposure to $\geq 5 \times 10^{-8}$ mol/L A23187 at 37 °C, significantly ($P < .01$) diminished release of LF by NG as compared with healthy AG suspensions was observed. In kinetics experiments using 10^{-7} mol/L A23187,

diminished release of LF by NGs was also evident following incubation intervals of >5 minutes ($P \leq .015$ as compared with healthy adult controls). In addition, moderately diminished exocytosis of vitamin B-12 transport protein, another secondary granule marker, was also apparent among six neonatal suspensions maximally stimulated with A23187 (2.5×10^{-7} mol/L, 20 minutes) ($P < .01$) (data not shown).

Similar but less striking differences between neonatal and control suspensions were evident in dose-response or kinetics experiments using PMA as a stimulant (Fig 2). Comparable LF release was observed under some experimental conditions, but diminished release by NGs was evident when they were exposed to high concentrations (100 or 500 ng/mL) of PMA for 20 minutes ($P < .05$). No significant ($P = .05$) differences were evident with respect to vitamin B-12 transport protein release among neonatal and healthy adult suspensions tested under the same conditions (data not shown). LF and vitamin B-12 transport protein release by Mac-1, LFA-1-deficient granulocyte suspensions was normal in response to both PMA and A23187 stimuli, as previously reported.¹³

Diminished LF release by NGs was also evident during



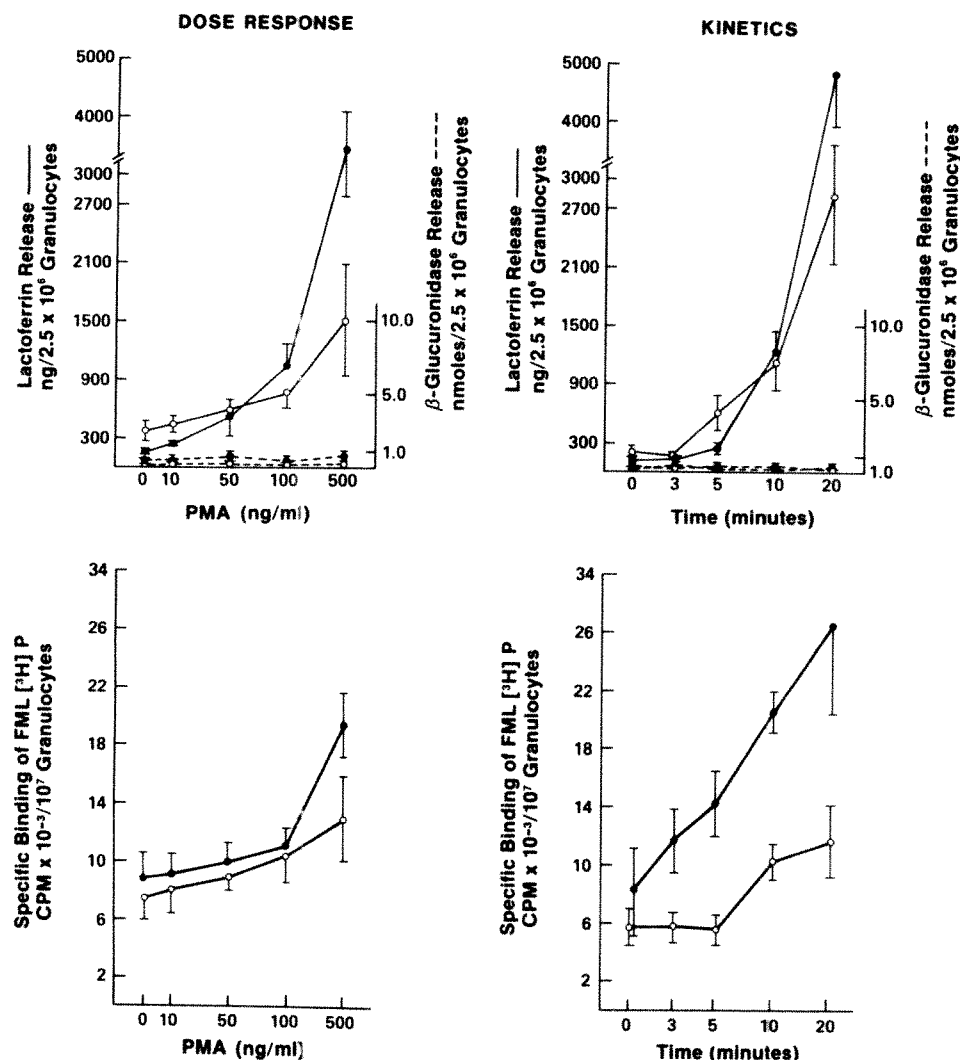


Fig 2. LF release and up-regulation of specific f-Met-Leu[³H]-Phe binding by NGs (cord blood) (○—○) or AGs (●—●) in response to PMA. Upper panels: LF release by NGs was comparable to healthy adult suspensions under most conditions, but diminished values for neonatal suspensions were evident when a maximal stimulus (500 ng/mL) was used for ≥ 20 minutes ($P < .05$). Lower left panel: Significantly ($P < .027$) diminished f-Met-Leu[³H]-Phe binding by NGs as compared with healthy adult suspensions was demonstrated following exposure to PMA (500 ng/mL, 20 minutes). In kinetics assessments (right lower panel) up-regulation of f-Met-Leu[³H]-Phe binding by AGs was evident following a 5-minute exposure to PMA (100 ng/mL), whereas a 10-minute exposure was required to enhance NG fML[³H]P binding significantly above baseline values. Values shown in all panels represent the mean $\pm$ 1 SD of individual determinations (adults, $n = 5$ to 11; neonates, $n = 3$ to 15, for each test condition).

cell spreading on glass surfaces either in the presence of chemotactic factors or secretagogues (data not shown). Mac-1, LFA-1-deficient granulocytes were used as experimental controls in these experiments because they fail to anchor but have a normal LF content.¹² Under all test conditions, neonatal and healthy adult suspensions demonstrated comparable anchorage or spreading following a 20-minute incubation interval at 21 °C. However, LF release during anchorage by stimulated neonatal cells was significantly diminished as compared with healthy adult cells [$(P \leq .05)$, and even lower values were observed for Mac-1, LFA-1-deficient cells] ($P < .0001$). Although fMLP (10^{-6} mol/L), and A23187 (2.5×10^{-7} mol/L), and PMA (500 μ g/mL) enhanced LF release by neonatal cells above baseline values, mean values in each case were significantly diminished as compared with healthy adult cells.

fMLP receptor induction. Induction of fMLP receptor expression was also assessed with respect to the same neonatal or control suspensions assayed for LF release, as shown in Figs 1 and 2. Methods previously described¹⁹ were used to quantitate specific, saturable binding of fML[³H]P before and following secretory stimulation. Binding studies were

carried out at 0 °C to prevent downregulation (pinocytosis) of fML[³H]P-receptor complexes. In dose-response experiments, diminished fML[³H]P binding by NGs was observed over a range of ionophore concentrations from $>2.5 \times 10^{-8}$ mol/L to 2.5×10^{-7} , and longer incubation intervals (>3 minutes) were required to up-regulate (enhance above baseline values) fML[³H]P binding by NGs as compared with healthy AG suspensions ($P \leq .05$). Among all experiments using maximal stimulating conditions, healthy AGs demonstrated a mean increase of specific binding of $410\% \pm 60\%$, whereas neonatal suspensions demonstrated a mean increase of $240 \pm 50\%$. Thus, both diminished induction of fMLP receptors and diminished release of LF were evident among neonatal suspensions stimulated in an identical fashion.

Somewhat similar relationships were observed when NGs or AGs were stimulated with PMA (Fig 2). Among both neonatal and healthy adult suspensions, concentrations of PMA ≥ 100 ng/mL elicited a significantly increased number of fML[³H]P binding sites as compared with baseline values. However, significantly ($P < .027$) enhanced fML[³H]P binding by adult cells as compared with neonatal

suspensions was demonstrated using PMA at a concentration of 500 ng/mL for 20 minutes. FMLP receptor up-regulation by adult cells was evident following a 5-minute exposure to PMA (100 ng/mL), whereas at least a 10-minute exposure was required to enhance NG fMLP [^3H]P binding significantly above baseline values. Under conditions of maximal PMA stimulation, healthy AGs demonstrated a mean increase of specific fMLP [^3H]P binding of $305\% \pm 70\%$, whereas neonatal suspensions demonstrated a mean increase of only $120\% \pm 30\%$ above baseline values.

Induction of surface Mac-1 α and β by chemotactic factors A23187 or PMA. The surface expression of Mac-1 α , LFA-1 α , their common β subunit, the complement receptor 1 (CR-1), and the HLA A₁B₁ antigen of neonatal or healthy AGs was evaluated using subunit-specific MAb to each antigen in immunofluorescence flow cytometry studies (Fig 3). Cell suspensions were studied prior to or following stimulation with fMLP (10^{-8} mol/L, 20 minutes), PMA (100 ng/mL, 20 minutes), or A23187 (10^{-7} mol/L, 20 minutes). Fluorescent histograms derived from these studies generally demonstrated a single population of cells among both adult and neonatal test suspensions. Under unstimulated conditions (DPBS), the mean surface expression values for each protein subunit were comparable among neonatal and healthy adult test groups ($P > .1$). Similar values were also determined when flow cytometric assessments were

performed on suspensions of leukocyte-rich plasma among neonates as compared with adult controls (data not shown). However, with respect to either fMLP, PMA, or A23187 (data not shown) stimuli, NGs generally demonstrated an impaired capacity to up-regulate the surface expression of Mac-1 α or β subunits. Mean fold increases of Mac-1 α or β expression were significantly diminished among neonatal suspensions as compared with adult suspensions assessed under comparable conditions (neonate—fMLP, 2.4 (Mac-1 α), 2.3 (β), PMA, 1.6 (Mac-1 α), 1.7 (β); healthy adult—fMLP, 5.5 (Mac-1 α), 4.9 (β), PMA, 4.9 (Mac-1 α), 4.8 (β) ($P < .04$ to $.001$). Individual values for stimulated Mac-1 α and/or β expression among neonatal suspensions were considerably less than healthy adult suspensions when compared in 17 of 22 experiments. As expected, no induction of LFA-1 α or HLA A₁B₁ by fMLP, PMA, or A23187 was evident among test or control suspensions, and the surface expression of these proteins was comparable among healthy adult and neonatal suspensions.

The capacity of chemotactic factors or PMA to up-regulate the surface expression of the CR-1 on NGs was also evaluated (Fig 4); such assessments were of particular interest since important differences between CR-1 and the CR-3 have been reported with respect to the anatomic location of their intracellular pools and their functional activities.^{13,14,24} For these studies, Mac-1, LFA-1-deficient

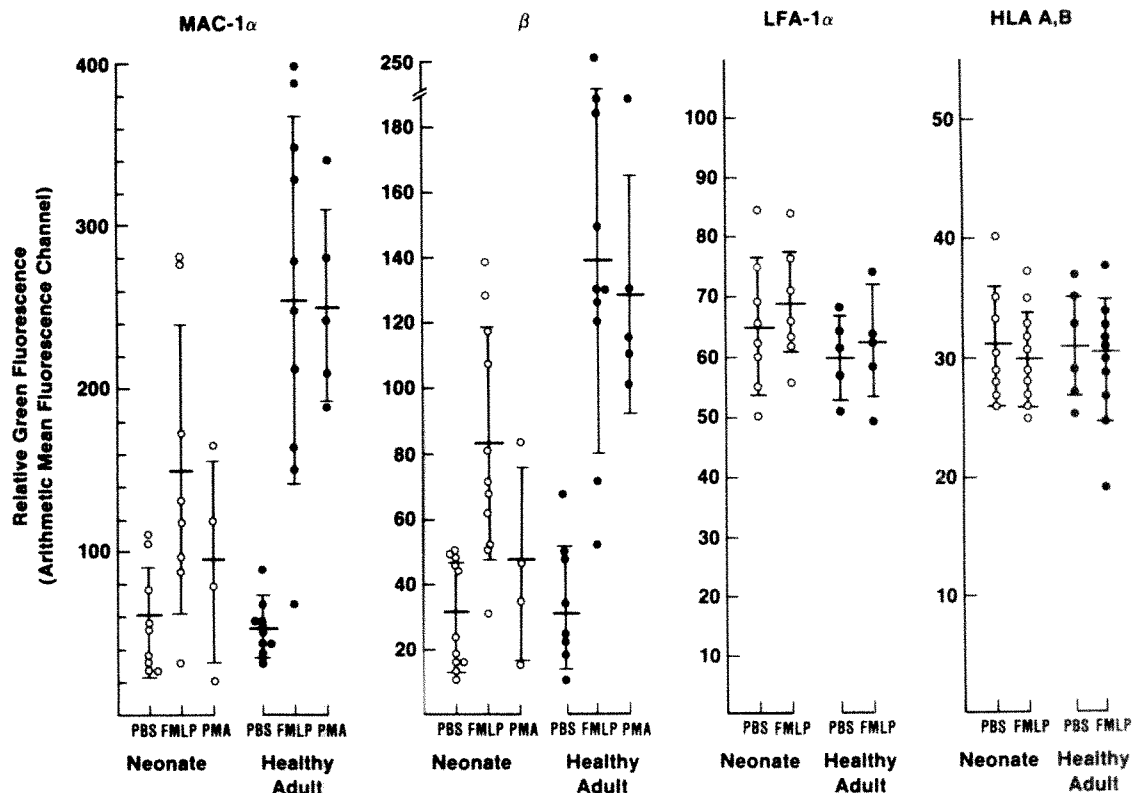


Fig 3. Expression of Mac-1 α , β , LFA-1 α , and HLA A,B, on NGs or healthy AGs as assessed with subunit specific monoclonal antibodies and immunofluorescence flow cytometry. NGs (○) or healthy AGs (●) were incubated in DPBS, fMLP (10 nmol/L) or PMA (100 ng/mL) for 20 minutes at 37 °C prior to antibody staining. Individual determinations and mean $\pm$ 1 SD values (bars) are shown for each test condition. Studies of neonatal suspensions included six cord blood and five peripheral blood samples.

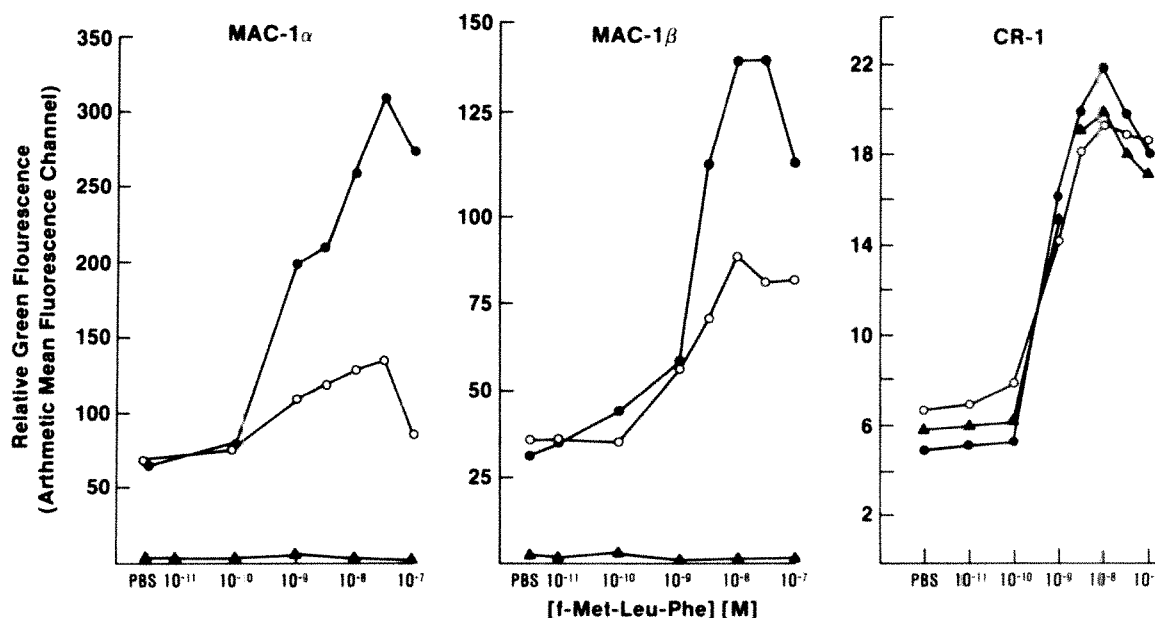


Fig 4. Expression of Mac-1 α , β , and CR-1 on NGs, Mac-1, LFA-1-deficient granulocytes, or healthy AGs as assessed by immunofluorescence flow cytometry. Neonatal (O), Mac-1, LFA-1-deficient (Δ), or healthy AGs ($\bullet$) were exposed to a range (0.01 to 10 nmol/L) of fMLP concentrations for 10 minutes at 21 °C prior to antibody staining. Values shown for each patient category represent the mean of five to nine separate determinations for each experimental condition. Neonatal suspensions assessed included four to six cord blood and two to four peripheral blood samples. Significant ($P \leq .001$) differences between healthy AG and NG Mac-1 α or β expression were observed using ≥ 1 nmol/L fMLP. Comparable baseline and stimulated expression of CR-1 was observed among each of the test groups under each test condition.

granulocytes were used as experimental controls because: (a) they express CR-1 normally, and (b) they contain and secrete (in suspension) normal quantities of specific granule constituents.¹

In contrast to Mac-1 α or β expression, no differences were evident among neonatal, healthy adult, and genetically deficient cell suspensions with respect to fMLP-mediated CR-1 expression (Fig 4). In contrast to their diminished capacity to express Mac-1 α or β , neonatal suspensions exposed to $\geq 10^{-9}$ mol/L fMLP expressed CR-1 normally. Mean fold increases of CR-1 expression (over baseline) following fMLP (10 nmol/L, 20 minutes) exposure were 3.1, 3.8, and 3.9 for neonatal, healthy adult, and Mac-1-deficient suspensions, respectively. Similar relationships were evident in dose-response experiments using C5a (1.5 to 20 μ g/mL concentration range), and normal CR-1 expression was also evident on NGs exposed to PMA (100 ng/mL, 20 minutes) (data not shown).

Relationship of Mac-1 glycoprotein expression to stimulated adherence properties. As illustrated in Fig 5, NGs, Mac-1-deficient granulocytes, and healthy AGs were stimulated with a range of concentrations of fMLP extending above and below that promoting optimal chemotaxis or hyperadherence.¹⁰ A sharp enhancement of adherence of healthy AGs was evident following their exposure to fMLP ($\geq 10^{-10}$ mol/L, 10 minutes, 21 °C). The correlation between percentage of enhancement of adherence and percentage of increase of Mac-1 α surface expression in response to a range of fMLP concentrations (10^{-11} to 10^{-8} mol/L) were highly significant ($r = .89$; $P < .001$). Although baseline (DPBS) adherence of neonatal granulocyte suspensions

was comparable to that of healthy adult cells as previously reported,¹ markedly diminished adherence increments were evident for each concentration of fMLP between 10^{-10} and 10^{-8} mol/L ($P \leq .001$). Adherence increments among stimulated neonatal and adult suspensions were directly related to relative enhancement of surface Mac-1 α or β expression. Similar relationships were observed for both healthy adult and neonatal suspensions in dose-response experiments using C5a or PMA as a stimulant (data not shown).

As shown in Fig 5, the degree of inhibition of adherence (percentage) by the anti-Mac-1 α MAb LM2.1 F(ab')₂ + the anti- β MAb TS1/18 F(ab')₂ was directly related to the mean level of stimulated adherence as well as the level of surface Mac-1 expression (as previously shown in Figs 3 and 4) on granulocytes among each test group. Significantly less ($P \leq .02$) inhibition of stimulated adherence was evident among neonatal suspensions as compared with healthy adult suspensions with respect to each fMLP concentration inclusive of 5×10^{-9} to 1×10^{-7} mol/L. As expected, no inhibitory effects were observed using Mac-1-deficient cells. Binding control antibodies including rabbit anti-CR-1 F(ab')₂ and anti-HLA A₁B₁ MAb demonstrated no inhibitory effects among neonatal, Mac-1-deficient or healthy adult suspensions (data not shown).

Ultrastructural evaluations: intracellular granule quantitation and morphometric determinations. No significant differences between unstimulated neonatal and healthy adult cells were evident with respect to the number of peroxidase-positive or peroxidase-negative granules per profile. However, neonatal cells treated with 1 nmol/L fMLP did not exhibit a significant decrease in peroxidase-negative

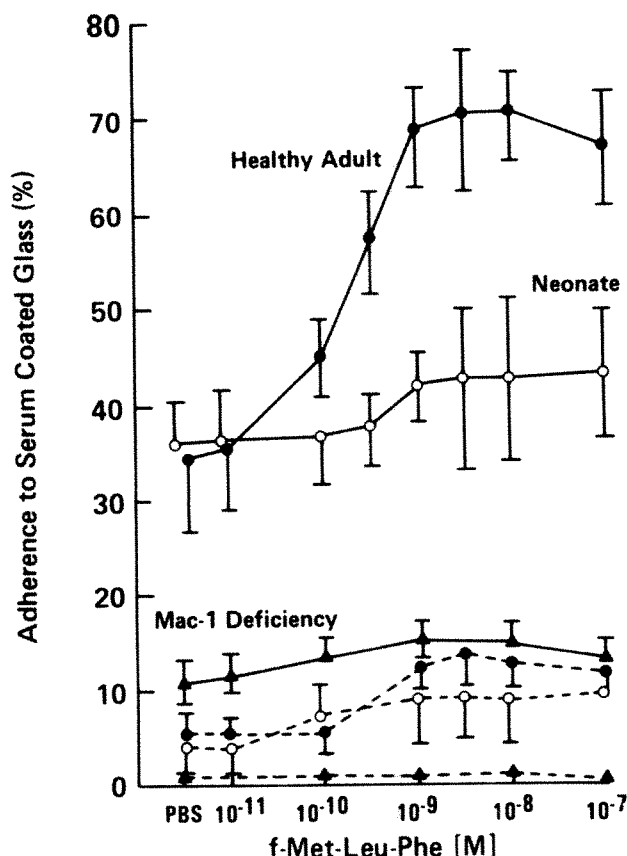


Fig 5. Adherence properties of NGs, Mac-1, LFA-1-deficient granulocytes, or healthy AGs with or without fMLP or anti-Mac-1 α + anti- β monoclonal antibodies. Granulocytes of each test group were exposed to a range of fMLP concentrations similar to that used in immunofluorescence flow cytometry experiments (Fig 4). As shown, the hyperadherence response of healthy AGs is observed over a similar range of stimulating concentrations that significantly enhance their surface expression of Mac-1 α and β (Fig 4). Although baseline adherence of neonatal suspensions was normal, adherence increments in response to fMLP were significantly diminished as compared with healthy adult values. Profoundly diminished baseline adherence and essentially no enhancement of adherence was observed using Mac-1-deficient granulocytes exposed to fMLP. Dashed lines demonstrate the effects of anti-Mac-1 α plus β preincubation on the adherence and hyperadherence responses of granulocytes of each patient population. Values shown represent the mean $\pm$ 1 SD of individual determinations in 7 healthy adults, 7 neonates, and 4 Mac-1 deficient patients.

granules, as was observed for healthy adult suspensions studied under these conditions (Table 3). Both neonatal and adult cells exposed to 100 nmol/L fMLP for 20 seconds did exhibit a significant reduction of peroxidase-negative granules, and no loss of peroxidase-positive granules was observed under these experimental conditions.

Morphometric evaluations of membrane perimeter and associated profile areas were also performed. Nonactivated neonatal neutrophils exposed significantly less plasma membrane (mean $\pm$ SD, $474 \pm 108 \mu^2$; S/V ratio, 1.19 ± 0.27 ; $n = 38$) than did nonactivated adult cells ($552 \pm 128 \mu^2$; S/V ratio, 1.39 ± 0.32 ; $n = 71$). Moreover, following activation with an optimum shape change inducing concentration of fMLP (1 nmol/L) for both neonatal and adult neutrophils,¹ the mean area determinations of neonatal and adult cells were comparable, but the exposed plasma membrane of newborn cells ($450 \pm 57 \mu^2$; S/V ratio, 1.13 ± 0.14 ; $n = 39$) was significantly ($P < .01$) less than that of stimulated adult cells ($648 \pm 194 \mu^2$; S/V ratio, 1.63 ± 0.49 ; $n = 42$). The increase in S/V ratio and calculated surface membrane caused by chemotactic stimulation of adult neutrophils were statistically significant ($P < .01$). There was no significant change in the neonate neutrophil. Thus, under at least selected experimental chemotactic conditions, neonatal neutrophils failed to demonstrate the peroxidase-negative granule loss and surface membrane increase seen in adult neutrophils.

DISCUSSION

These studies provide additional evidence that impaired chemotaxis of NGs is functionally linked to abnormalities of cellular effector mechanisms contributing to stimulated adherence (hyperadherence) properties.^{1-5,39,40} We previously observed impaired C5a, fMLP, or bacterial chemotactic factor-mediated hyperadherence of NGs and abnormal redistribution of their surface "adhesions sites" under conditions of sequential chemotactic factor stimulation and in response to colchicine.^{1,3} Krause and co-workers later reported abnormalities of granulocyte adherence in populations of healthy neonates⁴ and in "stressed" infants studied during the course of acute respiratory or cardiopulmonary illness.⁵ The molecular basis of these functional deficits has not been clearly elucidated. Defects of membrane deformability,⁴⁰ lectin binding and/or capping⁴¹ microtubule assembly,² and expression of membrane receptors for

Table 3. Quantitation of Granules in Healthy Adult or Neonatal Neutrophils: Effects of f-Met-Leu-Phe Stimulation

Experimental Conditions	Healthy Adult Neutrophil Granules*			Neonate Neutrophil Granules†		
	(n)	Peroxidase +	Peroxidase -	(n)	Peroxidase +	Peroxidase -
Baseline‡	40	74.2 $\pm$ 13.7§	60.2 $\pm$ 11.1	38	84.5 $\pm$ 15.4	59.1 $\pm$ 10.1
f-Met-Leu-Phe 1 nmol/L	22	72.6 $\pm$ 14.5	48.1 $\pm$ 9.8¶	40	80.6 $\pm$ 16.7	53.2 $\pm$ 13.2
f-Met-Leu-Phe 100 nmol/L	25	79.5 $\pm$ 11.3	48.2 $\pm$ 10.0¶	32	84.2 $\pm$ 12.2	50.2 $\pm$ 8.4¶

*Data pooled from five individuals.

†Data pooled from five individuals.

‡Cells incubated in DPBS for 5 minutes at 37°C prior to fixation.

§Mean $\pm$ SD: number of granules/profile (n = number of profiles examined).

||Cells incubated in DPBS for 5 minutes at 37°C, f-Met-Leu-Phe added, and incubation continued for an additional 20 seconds prior to fixation.

¶ $P < .01$ v baseline determination.

fMLP⁴² have been reported. Heterogeneity, including increased proportions of immature granulocyte forms⁶ or subpopulations of cells expressing less surface antigen identified by the 31D8 monoclonal antibody⁴³ in neonatal or cord blood samples may contribute to the observed abnormalities. Our studies were designed to evaluate in NGs the secretory processes that contribute to normal stimulated cellular adherence and/or migration.

The positively charged glycoprotein, LF, a known constituent of specific granules, appears to influence granulocyte-endothelial cell adherence interactions, homotypic granulocyte aggregation, and cell surface charge.²² Moreover, deficits of adherence and directed migration have been well documented in studies of patients with genetic deficiency of neutrophil-specific granules.^{20,21} Of considerable relevance to this report, Ambruso et al²⁶ previously documented profoundly diminished LF content in cord blood granulocytes, as well as diminished numbers and abnormal morphology of electron-dense (specific) granules. Our findings confirm diminished LF content of cord blood as well as peripheral blood granulocytes of healthy neonates. However, unlike Ambruso et al, we were unable to demonstrate quantitative morphologic abnormalities of peroxidase-negative granules in profiles of unstimulated cord blood or neonatal cells. Differences observed among these two studies may reflect the application of different ultrastructural techniques. Despite its diminished cellular content, LF released by neonatal cells under conditions of chemotactic stimulation was comparable to that of healthy adult cells in both studies. Indeed, in response to a chemotactically relevant range of fMLP concentrations, we observed that the percentage of LF released by neonatal samples was actually enhanced as compared with healthy adult controls.

Diminished LF release by NGs was clearly documented with secretagogue stimulation and during stimulated cell spreading, conditions that promoted overall greater granule depletion than chemotactic stimuli (Figs 1 and 2). Based on the previously determined number of LF molecules required to saturate granulocyte surfaces, it can be estimated that chemotactic conditions applied in these studies would promote sufficient LF release to saturate cell surfaces of both NGs and healthy AGs.⁴⁴ Thus, the relevance of diminished secretagogue-mediated LF release to diminished chemotactic function in neonates is uncertain. Assessments of LF release by NGs undergoing directed migration in Boyden chambers also demonstrated a comparable extent of release as compared with healthy adult cells (data not shown). Thus, although direct assessments of cell surface charge were not performed in these studies, it is unlikely that diminished LF cell surface deposition and consequent abnormalities of surface charge could account for abnormal stimulated adherence properties of NGs.

Previous reports have clearly documented that degranulating stimuli modulate the ability of the chemoattractant fMLP to bind to human neutrophils. Increased fMLP receptor expression and enhanced oxidative responsiveness to fMLP have been functionally related to preferential specific granule release.^{17,19} Such data support the concept that specific granules may represent a source of additional mem-

brane and chemoattractant receptors that can be integrated into the cell membrane at its leading edge during granule-membrane fusion associated with directed locomotion in vitro or exudation in vivo.¹⁷ Our findings suggest a limited capacity of NGs to up-regulate fMLP receptors in response to PMA or A23187 under the same experimental conditions demonstrating their diminished LF release. However, baseline fMLP receptor expression when studied at 0 °C was comparable among neonatal (cord blood) and healthy adult suspensions tested.¹ If LF and fMLP receptors are packaged in the same intracellular compartments and mobilized to cell surfaces in a similar manner, these latter findings would suggest that diminished LF content of NGs did not result from in vivo or artifactual in vitro specific granule release prior to our in vitro assessments. Further studies using fractionated granulocytes will be required to determine if abnormal surface expression results from diminished intracellular granular pools or as a result of abnormal translocation of fMLP receptors or other granule-associated constituents to cell surfaces. Our findings of diminished peroxidase-negative granule depletion under selected chemotactic conditions supports the latter possibility. These studies do not address the alternative possibility that NGs express only low-affinity as opposed to high-affinity fMLP receptors.^{45,46}

Our studies provide the first reported evidence that abnormal stimulated adherence and/or directed migration of NGs involves deficits of mobilization and/or surface expression of the adhesive protein, Mac-1 (iC3b receptor or CR-3). Mac-1, LFA-1, and p150,95 molecules constitute a family of structurally and functionally related human leukocyte surface glycoproteins that are critical determinants of cellular adhesive properties.⁴⁶ Inherited deficiency of these glycoproteins has been recognized in a number of patients with recurrent bacterial infection, persistent granulocytosis, and profoundly diminished leukocyte mobilization into extravascular inflammatory sites (reviewed in ref 53).¹²⁻¹⁴ Adherence-dependent functional defects of patient leukocytes have been reproduced by incubating normal cells with MABs directed at Mac-1 subunits.^{31,46-55} In unstimulated peripheral blood granulocytes, Mac-1 and p150,95 are present in an intracellular compartment as well as on the cell surface.^{13,14,17,32} Inflammatory mediators, including C5a and fMLP stimulate a three-to-seven-fold increase in Mac-1 and p150,95 on the cell surface. This up-regulation appears to be of great importance in regulating granulocyte and monocyte adhesiveness. Mac-1 has been shown to cosediment with secondary granules in fractionated granulocytes on sucrose gradients.²³ Impaired granulocyte hyperadherence and motility associated with genetic deficiency of Mac-1/LFA-1/p150,95 appears to be related directly to impaired induction of surface Mac-1 and p150,95 glycoproteins.^{13,14}

Our findings demonstrate significantly diminished surface expression of Mac-1 α , β , and p150,95 α (data not shown) on neonatal granulocytes exposed to chemotactic concentrations of fMLP, C5a (data not shown), as well as PMA and A23187 (data not shown). As also recently described by Bruce et al,⁵⁵ these deficits appear to be relatively specific for Mac-1 subunits since chemotactic induction of the CR-1

(C3b receptor) on NGs is normal. Such findings provide increasing evidence of important biologic differences between CR-1 and CR-3 with respect to function, intracellular anatomical location, and mechanisms regulating intracellular translocation and/or cell surface expression.²⁴ On the basis of our MAb-inhibition studies, our findings suggest that impaired hyperadherence of NGs is directly related to impaired Mac-1 α/β up-regulation. Findings of normal baseline expression and over-all greater levels of Mac-1 subunit expression on surfaces of NGs as compared with Mac-1, LFA-1-deficient granulocytes suggest pathogenic mechanisms related to intracellular translocation of Mac-1 and p150,95 rather than aberrant biosynthesis of these molecules in NGs. Direct assessments of intracellular Mac-1 pools using fractionated cells will be required to evaluate these possibilities further.

The implications of our findings with respect to deficits of

inflammation in human neonates are uncertain, but at least two distinct pathogenic mechanisms may be operative. Impaired chemotactic induction of the surface expression of Mac-1 or other adhesive molecules on NGs may contribute preferentially to disturbed cellular interactions with microvascular endothelium which, in turn, may diminish cellular localization at inflammatory sites. Alternatively, secretory abnormalities (lysosomal granule release, fMLP receptor up-regulation) recognized in our in vitro studies using relatively high stimulus conditions might be expected to contribute to deficits of motile, microbicidal, and/or cytotoxic functions of neonatal leukocytes infiltrating inflamed tissue sites. Confirmation and/or dissection of these or other in vivo inflammatory aberrations should be possible with applications of molecular probes (ie, anti-Mac-1 MAb) in studies of neonatal animal models.

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ADP-Induced Platelet Shape Change and Mobilization of Cytoplasmic Ionized Calcium Are Mediated by Distinct Binding Sites on Platelets: 5'-p-Fluorosulfonylbenzoyl adenosine Is a Weak Platelet Agonist

By A. Koneti Rao and Maria A. Kowalska

Platelet stimulation with ADP results in several responses, including shape change, increase in cytoplasmic ionized calcium concentration $[Ca^{2+}]_i$, and inhibition of adenylate cyclase. 5'-p-Fluorosulfonyl benzoyl adenosine (FSBA), which covalently labels an ADP binding site on platelets, blocks platelet shape change but not the inhibition of cyclic AMP levels by ADP, whereas p-chloromercuribenzenesulfonate (pCMBS), a nonpenetrating thiol reagent, has the opposite effects. We examined the effect of FSBA and pCMBS on ADP-induced increase in $[Ca^{2+}]_i$ using platelets loaded with fluorescent Ca^{2+} indicators quin2 and fura-2.

ADENOSINE DIPHOSPHATE (ADP) induces a number of responses in platelets, including shape change from disc to sphere, aggregation, and secretion of granule contents. It inhibits adenylate cyclase activity of platelet membranes^{1,2} and the accumulation of cyclic AMP in intact platelets induced by prostanooids such as prostaglandin E_1 (PGE_1).^{3,4} ADP does not penetrate the intact cell membrane,⁵ and the various responses are considered to be mediated by its interaction with specific binding sites on the platelet surface. The interaction of ADP with the putative membrane binding sites has been probed using several compounds, including ATP, ADP analogs, and the thiol reagent, p-chloromercuri benzenesulfonate (pCMBS).⁶ Platelet shape change and the antagonism of adenylate cyclase activity by ADP are inhibited by ATP.⁷ The ADP analogs, 2-azido-ADP and 2-methylthio-ADP, are three- to fivefold more potent than ADP as aggregating agents, but 10 to 200-fold more potent as inhibitors of PGE_1 -stimulated cyclic AMP accumulation in intact platelets.^{8,9} In contrast, the nonpenetrating, mercurial thiol reagent pCMBS does not inhibit shape change but blocks the effect of ADP on adenylate cyclase¹⁰ and antagonizes the platelet binding of both the above-mentioned ADP analogs.^{8,9}

5'-p-Fluorosulfonylbenzoyl adenosine (FSBA) interacts covalently with nucleophilic residues in the region of ADP or ATP binding sites on enzymes and platelets.^{11,12} It is covalently incorporated into a 100-kilo Dalton (kD) membrane protein in intact platelets and inhibits ADP-induced shape change, aggregation, and fibrinogen binding, but does not block the inhibitory effect of ADP on cyclic AMP accumulation.^{13,14} Furthermore, FSBA does not block the platelet binding of 2-methylthio-ADP.¹⁴ Based on evidence from studies with ADP antagonists and analogs,^{6,8,9} platelets have been proposed to possess at least two different types of binding sites for ADP, one mediating the shape change and the second the inhibition of adenylate cyclase.

Platelet stimulation with ADP results in an increase in the cytoplasmic ionized calcium concentration $[Ca^{2+}]_i$, which plays an important role in platelet activation.¹⁵ Using quin2¹⁶ and fura-2¹⁷ as fluorescent indicators of changes in $[Ca^{2+}]_i$, we examined the effect of FSBA and pCMBS on ADP-induced changes in $[Ca^{2+}]_i$. Our studies suggest that the rise in $[Ca^{2+}]_i$ and the inhibition of adenylate cyclase following

FSBA (50 to 200 $\mu\text{mol/L}$) induced a dose-dependent rise in $[Ca^{2+}]_i$, indicating that it is a weak platelet agonist. Under conditions of covalent labeling of the ADP binding sites, FSBA (50 to 100 $\mu\text{mol/L}$) did not inhibit the ADP-induced increase in $[Ca^{2+}]_i$ or its inhibition of adenylate cyclase, whereas pCMBS (up to 1 mmol/L) abolished both these responses but not shape change. These findings suggest that ADP-induced Ca^{2+} mobilization and inhibition of adenylate cyclase are mediated by platelet binding sites distinct from those mediating shape change.

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platelet stimulation with ADP occur through binding sites or membrane-related mechanisms that are distinct from those related to shape change. We demonstrate that FSBA by itself induces an increase in $[Ca^{2+}]_i$, indicating that it behaves as a platelet agonist.

MATERIALS AND METHODS

Materials. Quin2 acetoxymethyl ester, fura-2 ester, and ionomycin were obtained from Calbiochem (San Diego) and were stored in 100% dimethylsulfoxide (DMSO) (Aldrich, Milwaukee) at -20°C over desiccant. FSBA (Sigma Chemical, St Louis) solutions were freshly prepared in dimethylformamide (DMF) and the concentration was determined spectrophotometrically using extinction coefficient of $1.35 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at 259 nm. ADP, pCMBS, adenosine deaminase, hirudin, apyrase, and theophylline were purchased from Sigma. All other chemicals were of the highest grade available commercially. The stable prostaglandin I_2 derivative, ZK 36374¹⁸ was kindly donated by Berlex Laboratories, Cedar Knolls, NJ.

Methods. Blood was drawn from healthy volunteers who had not ingested aspirin for at least 10 days and was mixed with one-sixth vol of acid-citrate-dextrose.¹⁹ Platelet-rich plasma (PRP) was prepared by centrifugation for 15 minutes at 150 g at room temperature.

Studies with quin2 and fura-2. The platelets (3×10^8 plat/mL) were loaded with quin2 by incubating 20 mL of PRP with 20 μL of

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quin2 ester (20 $\mu\text{mol/L}$ final concentration) at 37°C for 30 minutes. Final concentration of DMSO was 0.1%. The suspension was then centrifuged at 1,000 g for 15 minutes at room temperature. Hirudin (0.05 U/mL, final concentration) and apyrase (10 $\mu\text{g/mL}$, final concentration) were added prior to centrifugation to prevent platelet activation.²⁰ The sedimented platelets were resuspended to a concentration of 2×10^8 cells/mL in HEPES buffer, pH 7.4 (HEPES 10 mmol/L, NaCl 145 mmol/L, KCl 5 mmol/L, MgSO_4 1 mmol/L, glucose 0.1%) in the presence of apyrase (10 $\mu\text{g/mL}$, final concentration). Before each experiment, the cells (2-mL aliquots) were equilibrated at 37°C for 3 minutes. The external calcium concentration was adjusted by addition of 10 μL of 200 mmol/L CaCl_2 (1 mmol/L final concentration) followed by incubation for 2 minutes at 37°C. During the experiments, 2-mL aliquots of platelet suspensions were continuously stirred in a quartz cuvette (Fisher, Springfield, NJ) thermostatted at 37°C. Small aliquots (5 to 10 μL) of agents were added, and fluorescence was recorded over time. The fluorescence signal was recorded at 492 nm (excitation at 339 nm/L) using a Perkin Elmer LS-5 Spectrophotofluorimeter (Oatbrook, IL). The calibration of the fluorescence signal as a function of Ca^{2+} was performed as described by Tsien and co-workers¹⁶ based on the equation:

$$F = F_{\min} + \frac{(F_{\max} - F_{\min})(\text{Ca}^{2+}/\text{kd})}{1 + \text{Ca}^{2+}/\text{kd}}$$

where $F_{\min}$ and $F_{\max}$ represent fluorescence at a very low and a very high calcium concentration, respectively. For estimation of $F_{\max}$, we added a high concentration of ionomycin (400 to 800 nmol/L) in the presence of 1 mmol/L external Ca^{2+} .²¹ $F_{\min}$ was calculated as follows: $F_{\min} = AF + (F_{\max} - AF)/6$, where AF is autofluorescence of the cells after the addition of 1 mmol/L MnCl_2 to quench the dye fluorescence.²¹

For experiments with fura-2, the PRP was incubated with fura-2 ester (5 $\mu\text{mol/L}$ final concentration) for 30 minutes at 37°C and resuspended in HEPES buffer as described above for quin2-loaded platelets. Binding of Ca^{2+} to fura-2 results in a shift in peak excitation wave length from 380 to 340 nm. The $[\text{Ca}^{2+}]_i$ were calculated as described by Grynkiewicz et al¹⁷ based on the ratio of fluorescence emission (510 nm) following excitation at 340 and 380 nm.

Treatment of platelets with FSBA or pCMBS. All experiments with FSBA were carried out in the presence of 2 U/mL adenosine deaminase to degrade the contaminating adenosine, which inhibits platelets.^{22,23} FSBA is covalently incorporated into intact platelet membranes in a time-dependent and saturable manner with 80% of maximal incorporation by 30 minutes.¹³ Therefore, platelets were incubated for 30 minutes at 37°C with the analog to permit covalent labeling of the ADP binding site.^{13,14} For experiments with pCMBS, platelet suspensions were incubated for 2 minutes because of its rapid onset of effect. In experiments involving measurement of $[\text{Ca}^{2+}]_i$ levels, platelets were loaded with quin2 or fura-2 prior to treatment with FSBA or pCMBS.

Studies on shape change and accumulation of cyclic AMP. Platelet suspensions were prepared from PRP as described above. Platelet shape change was studied in the presence of 2 mmol/L EDTA and was recorded using a Chrono-Log Aggregometer (Chrono-Log, Havertown, PA) as a change in optical density.

The studies on the accumulation of cyclic AMP were performed by incubating platelets, pretreated with either FSBA for 30 minutes or pCMBS for 2 minutes, with the stable prostaglandin I_2 derivative, ZK 36,374 (50 nmol/L) for 2.5 minutes at 37°C in the presence of theophylline (7 mmol/L). Various concentrations of ADP were then added, and platelets were incubated for an additional 2.5 minutes before extraction with 20% trichloroacetic acid (TCA). The cyclic AMP levels were measured using a radioimmunoassay²⁴ (RIANEN

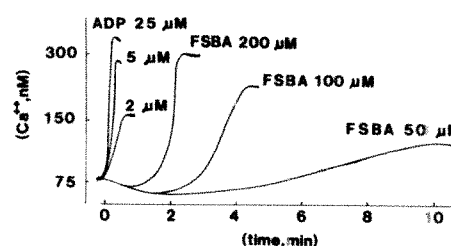


Fig 1. Effect of ADP and FSBA on $[\text{Ca}^{2+}]_i$. Aliquots (2 mL) of platelet suspensions (2×10^8 /mL) loaded with quin2, were taken in the cuvette and equilibrated with stirring at 37°C for 3 minutes and then with 1 mmol/L external Ca^{2+} for an additional 2 minutes. ADP or FSBA were added in the presence of adenosine deaminase (2 U/mL). The fluorescence recordings are shown. Shown on the ordinate are the $[\text{Ca}^{2+}]_i$ calculated from the fluorescence as described in the Materials and Methods section. The experiments shown in this and subsequent Figs 2 through 8 are representative of at least four separate experiments performed with platelets from different donors.

platelets with increasing concentrations of FSBA in the presence of adenosine deaminase. In this experiment, platelet-cAMP KIT, Dupont, Wilmington, DE) in the supernatants after lyophilization.

RESULTS

Effect of ADP and FSBA on cytoplasmic ionized calcium concentration. Addition of ADP to quin2-loaded platelets resulted in a prompt increase in $[\text{Ca}^{2+}]_i$ (Fig 1) from a basal level of ~ 80 nmol/L to peak levels of 270 ± 91 nmol/L (mean $\pm$ SD) and 292 ± 102 nmol/L on stimulation with 5 and 25 $\mu\text{mol/L}$ ADP, respectively ($n = 22$). With FSBA at concentrations > 50 $\mu\text{mol/L}$ and in the presence of adenosine deaminase (2 U/mL), there was a dose-dependent rise in $[\text{Ca}^{2+}]_i$, which was slower than that noted with ADP. Adenosine deaminase by itself did not induce any changes in $[\text{Ca}^{2+}]_i$. In the absence of adenosine deaminase, the rise in $[\text{Ca}^{2+}]_i$ on addition of FSBA was lower (not shown); this is in line with previous studies¹⁴ demonstrating a rapid platelet inhibitory effect of FSBA due to the presence of adenosine in the FSBA preparations.

Figure 2 shows the $[\text{Ca}^{2+}]_i$ in response to incubation of

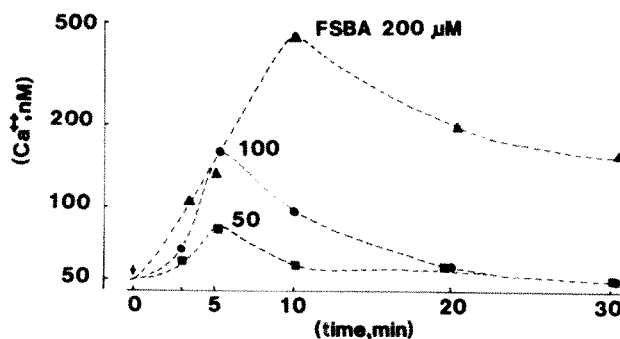


Fig 2. Effect of FSBA on $[\text{Ca}^{2+}]_i$. Platelet suspensions, loaded with quin2, were incubated without stirring at 37°C with increasing concentrations of FSBA. At various time intervals, 2-mL aliquots were taken in the cuvette, external Ca^{2+} was equilibrated to 1 mmol/L with CaCl_2 , and fluorescence signals were recorded. Shown are the peak $[\text{Ca}^{2+}]_i$ at various time points.

lets were incubated at 37°C with FSBA without stirring; at various intervals $[Ca^{2+}]_i$ were measured in aliquots after equilibration with $CaCl_2$ (1 mmol/L). With 50 to 200 $\mu\text{mol/L}$ FSBA, there was a slow, dose-dependent increase in the $[Ca^{2+}]_i$, followed by a gradual return to lower levels over 20 to 30 minutes. No such increase was noted at FSBA concentrations $<50 \mu\text{mol/L}$. To demonstrate that the increase in fluorescence noted with FSBA reflected platelet activation and increase in $[Ca^{2+}]_i$, we studied the effect of a stable PGI_2 analog, ZK 36,374 (0.5 $\mu\text{mol/L}$) on the responses induced by FSBA. Previous studies have demonstrated that the rise in $[Ca^{2+}]_i$ on platelet stimulation is inhibited by agents that elevate platelet cAMP.²⁵ The rise in $[Ca^{2+}]_i$ noted with FSBA and ADP were completely inhibited (not shown) by prior exposure of platelets to 0.5 $\mu\text{mol/L}$ ZK 36,374 in the presence of theophylline (7 mmol/L). These studies provide direct evidence that FSBA activates platelets and leads to a rise $[Ca^{2+}]_i$. However, the rise was slower than with ADP and, in general, the peak $[Ca^{2+}]_i$ were comparable to those achieved with about a tenfold lower concentration of ADP, indicating that FSBA is a weak agonist.

Effect of FSBA on ADP-induced increase in cytoplasmic ionized calcium concentration and shape change. Studies with ^3H -sulfonylbenzoyl adenosine indicate that maximal incorporation into intact platelets occurs by 60 minutes, with 80% incorporation by 30 minutes.¹³ Previous studies^{13,14} using FSBA as an inhibitor of ADP-induced responses have been performed with incubations of 30 to 60 minutes. Incubation of platelets with FSBA (25, 50 $\mu\text{mol/L}$) for 30 minutes at 37°C abolished ADP (5 $\mu\text{mol/L}$) induced shape change but did not inhibit the rise in $[Ca^{2+}]_i$ (Fig 3). However, with PAF (100 nmol/L), FSBA did not inhibit either shape change or the calcium responses (Fig 3).

To determine the effect of FSBA at various intervals of incubation, platelet suspensions were incubated at 37°C with 100 $\mu\text{mol/L}$ FSBA without stirring, and the response to ADP (5 $\mu\text{mol/L}$) was recorded at different intervals in separate aliquots (Fig 4). When $[Ca^{2+}]_i$ prior to addition of

ADP was lower than the peak value induced by FSBA alone, the increment in $[Ca^{2+}]_i$ signal due to the ADP was clearly demonstrable. As the $[Ca^{2+}]_i$ returned close to basal levels, addition of ADP evoked a rise in $[Ca^{2+}]_i$, comparable to that in control platelets incubated at 37°C for 30 minutes with DMF. Following a 30-minute incubation with FSBA, the peak calcium levels on ADP stimulation were only minimally decreased as compared with those in platelets incubated with DMF alone. These studies indicate that under conditions at which FSBA completely inhibits ADP-induced shape change,^{13,14} it does not abolish ADP-induced increase in $[Ca^{2+}]_i$.

Studies were also performed using other agonists, PAF (40 nmol/L) and thrombin (0.1 U/mL) (Fig 4). The results were similar to those with ADP in that the peak $[Ca^{2+}]_i$ at various time intervals were comparable in the presence or absence of FSBA. These results also indicate that the increment in $[Ca^{2+}]_i$ noted on stimulation of platelets at elevated initial $[Ca^{2+}]_i$ is not the same as that following stimulation at basal levels. At 30 minutes of incubation with FSBA, the peak levels on stimulation were minimally decreased as compared with that in control platelet incubated with DMF.

In some experiments, the effect of FSBA was studied using platelets loaded with fura-2, a newer Ca^{2+} indicator that offers a much greater fluorescence than quin2 and obviates some of its drawbacks.¹⁷ Incubation of fura-2-loaded platelets with FSBA (50 and 100 $\mu\text{mol/L}$) resulted in a rise in $[Ca^{2+}]_i$ similar to that noted in quin2 loaded platelets (Fig 5). Moreover, following 30-minute incubation with FSBA, the responses to ADP (25 $\mu\text{mol/L}$) were not abolished and were similar to those in platelets incubated with DMF alone. On stimulation with ADP, the rise in

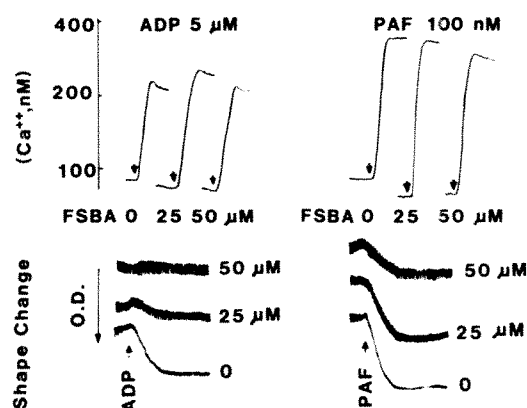


Fig 3. Effect of FSBA on the rise in $[Ca^{2+}]_i$ and shape change induced by ADP and PAF. Platelet suspensions were incubated for 30 minutes at 37°C with FSBA. The Ca^{2+} responses were monitored in the presence of 1 mmol/L external Ca^{2+} . The shape change was recorded in the presence of EDTA (2 mmol/L) using a Chrono-Log aggregometer.

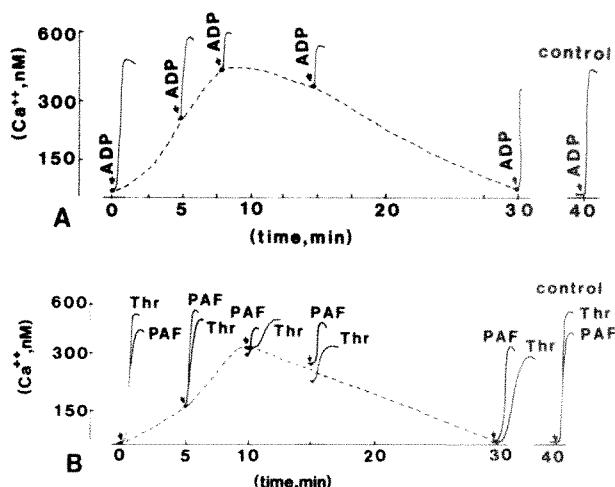


Fig 4. Effect of FSBA on the increase in $[Ca^{2+}]_i$ induced by (A) ADP, and (B) platelet-activating factor (PAF) and thrombin (Thr). Quin2-loaded platelet suspensions were incubated with FSBA (100 $\mu\text{mol/L}$) at 37°C without stirring. At various time intervals, aliquots (2 mL) were placed in the cuvette, equilibrated with 1 mmol/L external Ca^{2+} , and stimulated with ADP (25 $\mu\text{mol/L}$), thrombin (0.1 U/mL), or PAF (40 nmol/L) while being stirred; fluorescence signals were recorded. For controls, platelet suspensions were incubated for 30 minutes at 37°C with DMF (0.2%) and the responses were recorded. $[Ca^{2+}]_i$ prior to addition of the agonist (...).

$[Ca^{2+}]_i$ was higher in platelets incubated with 50 $\mu\text{mol/L}$ FSBA than with 100 $\mu\text{mol/L}$ FSBA, whereas the $[Ca^{2+}]_i$ prior to ADP stimulation were higher with the latter. These findings support the conclusions from experiments with quin2-loaded cells that FSBA does not inhibit the rise in $[Ca^{2+}]_i$ induced by ADP.

Effect of pCMBS on ADP and FSBA-induced increase in cytoplasmic ionized calcium concentration and on ADP-induced shape change. Incubation of quin2-loaded platelets with pCMBS (up to 1 mmol/L) alone did not result in an increase in the basal $[Ca^{2+}]_i$. Following a 2-minute incubation with pCMBS, the peak responses to ADP (25 $\mu\text{mol/L}$) were strikingly inhibited in a dose-dependent manner (Fig 6) with >50% inhibition at pCMBS concentrations >5 $\mu\text{mol/L}$. Because of the slower rise in peak $[Ca^{2+}]_i$ with FSBA, the effect of pCMBS was studied on the $[Ca^{2+}]_i$ at 4 minutes after addition of FSBA (100 $\mu\text{mol/L}$). Incubation for 2 minutes with pCMBS inhibited the rise in $[Ca^{2+}]_i$, although the inhibition required higher pCMBS concentrations than with ADP. In contrast, pCMBS (1 mmol/L) did not inhibit ADP-induced shape change (Fig 7).

Effect of FSBA and pCMBS on ADP-induced inhibition of cyclic AMP levels. Platelet suspensions preincubated with FSBA (100 $\mu\text{mol/L}$) for 30 minutes or with pCMBS (1 mmol/L) for 2 minutes were incubated with ZK 36,374 (50 nmol/L) for 2.5 minutes in the presence of theophylline (7 mmol/L) and then with increasing ADP concentrations for 2.5 minutes. The inhibition of cyclic AMP levels by ADP in platelets incubated with FSBA was comparable to that in platelets incubated with buffer or DMF (Fig 8). Thus, FSBA did not impair the ability of ADP to inhibit adenylate cyclase. The peak levels of cyclic AMP following exposure to ZK 36,374 (but before exposure to ADP) were lower (217 pmol/ 10^8 platelet, mean of two experiments in duplicate) in platelets preincubated with FSBA for 30 minutes than in platelets exposed to DMF alone (270 pmol/ 10^8 platelet); this

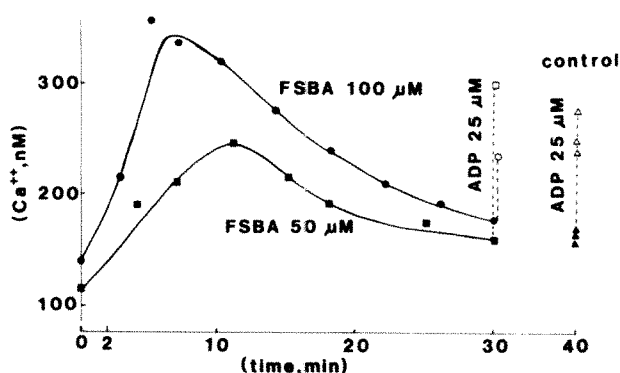


Fig 5. Effect of FSBA on $[Ca^{2+}]_i$ in platelets loaded with fura-2. An aliquot (2 mL) of platelet suspensions loaded with fura-2 was incubated with FSBA (50 or 100 $\mu\text{mol/L}$) at 37°C with stirring; fluorescence was recorded at various time intervals. At 30 minutes, ADP (25 $\mu\text{mol/L}$) was added, and fluorescence was recorded. Control platelets were incubated with DMF (0.2%) for 30 minutes prior to addition of ADP; these responses are shown in triplicate. Shown are the $[Ca^{2+}]_i$ levels calculated from the ratio of fluorescence recorded at 340 and 380 nm (excitation; emission 510 nm) as described in the Materials and Methods section.

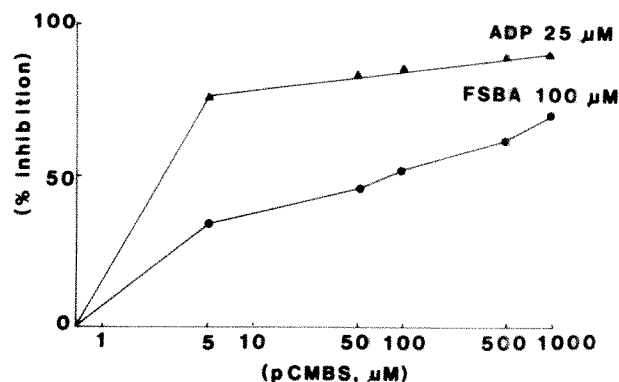


Fig 6. Effect of pCMBS on changes in $[Ca^{2+}]_i$ induced by ADP (25 $\mu\text{mol/L}$) and FSBA (100 $\mu\text{mol/L}$). Quin2-loaded platelet suspensions (2 mL) were equilibrated at 37°C for 3 minutes and then with 1 mmol/L external Ca^{2+} and pCMBS (or buffer) for an additional 2 minutes with stirring. ADP (25 $\mu\text{mol/L}$) or FSBA (100 $\mu\text{mol/L}$) was added, and the fluorescence was recorded. Shown is the inhibition in $[Ca^{2+}]_i$ recorded at 1 minute after ADP and at 4 minutes after addition of FSBA.

reflects the effect of FSBA as a weak agonist in partially inhibiting cAMP levels as well. Preincubation with pCMBS (1 or 100 $\mu\text{mol/L}$) for 2.5 minutes abolished the effect of ADP in inhibiting the accumulation of cyclic AMP (Fig 8).

DISCUSSION

FSBA is a nonpenetrating, adenosine nucleotide affinity analog which covalently modifies only a single polypeptide chain (mol wt 100,000) in intact platelets.¹³ It has been used effectively as an antagonist to examine the various ADP-induced platelet responses, including shape change, aggregation, and fibrinogen binding.^{13,14,26,27} We demonstrate that FSBA (50 to 200 $\mu\text{mol/L}$) by itself induces a concentration-dependent increase in $[Ca^{2+}]_i$ (Figs 1 and 2) and provide direct evidence that FSBA is a platelet agonist. The agonist role of FSBA is supported by several lines of evidence. At higher concentrations, FSBA induces shape change,²⁸ myosin phosphorylation²⁸ and thromboxane A_2 synthesis in platelets (Rao AK, Kowalska A, Disa J, unpublished observations). These effects occurred at concentrations higher than those at which we demonstrate mobilization of intracellular Ca^{2+} . Nevertheless, FSBA is a weak activator of platelets; the concentrations that yield comparable increases in $[Ca^{2+}]_i$ are about tenfold higher than those for ADP (Fig 1).

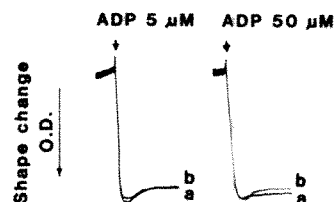


Fig 7. Effect of pCMBS on ADP-induced shape change. Platelets were preincubated with buffer (a) or pCMBS (1 mmol/L, b) for 2 minutes at 37°C. Aliquots were then stimulated with ADP (5 or 50 $\mu\text{mol/L}$) in the presence of EDTA (2 mmol/L). Shape change was recorded using a Chrono-Log aggregometer.

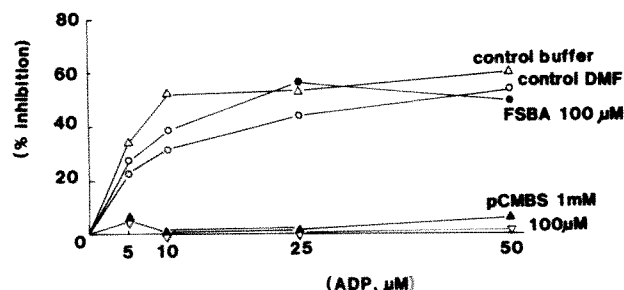


Fig 8. Effect of FSBA and pCMBS on ADP-induced inhibition of cyclic AMP levels. Platelets ($3 \times 10^8/\text{mL}$) were preincubated for 2 minutes with pCMBS (1 or 100 $\mu\text{mol/L}$) or for 30 minutes with FSBA (100 $\mu\text{mol/L}$) at 37°C and with ZK 36,374 (50 nmol/L) in the presence of theophylline (7 mmol/L) for 2.5 minutes. Platelets were then incubated with ADP for 2.5 minutes, and the reaction was stopped by addition of TCA (10% final concentration). The supernatants were lyophilized, and cAMP levels were measured.²⁴

In agreement with previous studies,¹⁴ FSBA abolished ADP-induced shape change but did not inhibit the effect of ADP on PGI_2 -stimulated cyclic AMP accumulation (Figs 3 and 8). Under conditions that permit covalent labeling of the putative ADP binding sites, FSBA (100 $\mu\text{mol/L}$) failed to inhibit ADP-stimulated increase in $[\text{Ca}^{2+}]_i$ in platelets loaded with quin2 (Figs 3 and 4) or fura-2 (Fig 5). As expected, incubation with FSBA (100 $\mu\text{mol/L}$) did not abolish the rise in $[\text{Ca}^{2+}]_i$ induced by PAF or thrombin. With all three agonists studied, the peak responses following the 30-minute incubation with FSBA were minimally reduced as compared with platelets incubated with DMF (Fig 4). We have observed similar findings in other experiments (not shown) in which platelets were stimulated with a second agonist (eg, thrombin) after an interval (10 to 30 minutes) following initial exposure to a different agonist (ADP or PAF); the peak responses to the second agonist were somewhat blunted as compared with those elicited by the second agonist alone. Thus, the small reduction in the peak $[\text{Ca}^{2+}]_i$ levels noted following platelet incubation with FSBA may reflect incomplete return to the basal state after activation by FSBA. Overall, we interpret these findings to indicate that FSBA does not inhibit ADP-induced elevation in $[\text{Ca}^{2+}]_i$ and that the rise in $[\text{Ca}^{2+}]_i$ induced by ADP is mediated by binding sites on platelets distinct from those mediating shape change.

The above conclusions are supported by the studies using pCMBS. This reagent blocked ADP-induced inhibition of cyclic AMP accumulation (Fig 8), but not shape change in platelets (Fig 7). However, at strikingly lower concentrations, pCMBS blocked the rise in $[\text{Ca}^{2+}]_i$ (Fig 6). These findings suggest also that the effect of ADP on cyclic AMP levels and the rise in $[\text{Ca}^{2+}]_i$ are mediated by the same binding site on platelets. The distinct characteristics of the two types of putative ADP binding sites on platelets is also supported by observations that FSBA does not inhibit the platelet binding of 2-methylthio-ADP,¹⁴ whereas pCMBS does.⁹ Compared with ADP, 2-methylthio-ADP is three to five times as active as an aggregating agent, but 150 to 200

times as potent as an inhibitor of cyclic AMP accumulation.⁹

Although the divergence in the effects of various ADP antagonists and analogs on platelet responses has been interpreted^{6,8,9} as evidence for the existence of different types of ADP binding sites, the data do not exclude other possibilities. For example, a single type of binding site could be coupled to one or other pathways under different conditions, or to both simultaneously if a common protein (eg, a guanine nucleotide-binding protein) mediates both actions. However, the model invoking the existence of two discrete binding sites provides a simpler explanation for several of the observations. Irrespective of the number of specific ADP binding sites, our studies clearly indicate the existence of distinct membrane-related mechanisms by which ADP induces the different platelet responses. The definitive subdivision of ADP binding sites awaits development of potent and selective inhibitors as has been possible with adrenergic receptors on tissues.

Our studies suggest that FSBA has a weak effect at the ADP binding site mediating the Ca^{2+} signals as well. First, FSBA by itself induced a rise in $[\text{Ca}^{2+}]_i$ that was inhibited by pCMBS (Fig 6) and second, it had a weak inhibitory effect on cAMP accumulation induced by ZK 36,374. The finding that FSBA acts on both types of binding sites is not unexpected given the structural similarities between FSBA and ADP, and is in accord with observations with other ADP analogs, 2-methylthio-ADP⁹ and 2-azido-ADP,⁸ which also produce effects indicative of interaction with both types of ADP binding sites, albeit with striking differences in their potencies.

Our studies were performed using quin2 and fura-2 as the $[\text{Ca}^{2+}]_i$ indicators. Another indicator that has been used to study Ca^{2+} mobilization in platelets is the Ca^{2+} -sensitive photoporphyrin, aequorin.²⁹ It has been suggested²⁹ that these indicators detect different pools of $[\text{Ca}^{2+}]_i$ within the platelets and that aequorin may have the capability of more sensitively detecting localized elevations in $[\text{Ca}^{2+}]_i$ not revealed by quin2, which reflects the average $[\text{Ca}^{2+}]_i$. Therefore, we cannot make conclusions on the relationship between changes in $[\text{Ca}^{2+}]_i$ and the platelet responses until similar studies are performed with aequorin.

In summary, we have provided evidence that FSBA by itself behaves as a weak platelet agonist. We have demonstrated that (a) FSBA inhibits ADP-induced shape change but does not block either elevation in $[\text{Ca}^{2+}]_i$ or inhibition of cyclic AMP levels by ADP; (b) the thiol reagent, pCMBS inhibits ADP-stimulated increase in $[\text{Ca}^{2+}]_i$, and its effect on adenylate cyclase but not shape change. The evidence presented is in accord with the conclusion that ADP-induced elevation in $[\text{Ca}^{2+}]_i$ is mediated by binding sites distinct from those leading to shape change but are probably the same as those mediating inhibition of cyclic AMP accumulation.

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Defensin-Rich Dense Granules of Human Neutrophils

By William G. Rice, Tomas Ganz, Joseph M. Kinkade, Jr, Michael E. Selsted, Robert I. Lehrer, and Richard T. Parmley

Defensins are a newly recognized class of small, cationic polypeptides that have *in vitro* microbicidal activity toward certain bacteria, fungi, and viruses. Human neutrophil granules were separated into 13 density fractions by using a high-resolution Percoll gradient centrifugation procedure, and the distribution of the three defensin polypeptides in these fractions was determined. Levels of defensins and several granule marker proteins were estimated in each fraction from relative staining intensities of bands following acid-urea and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total acid-extractable proteins. These results were confirmed by enzyme immunoassay measurements of defensins and quantitative determinations of the typical azurophil granule components, myeloperoxidase, β -glucuronidase, lysozyme, and elastase. The five higher density granule fractions (H1 through H5) contained fourfold higher relative amounts of defensins as compared with the eight lower density fractions (L1 through L8), accounting for ~50% of the total

protein. In particular, fraction H5 was especially enriched in defensins but was relatively deficient in myeloperoxidase, β -glucuronidase, lysozyme, and elastase. Ultrastructural morphology showed that fraction H5 contained the largest granules. Seventy percent of these granules exhibited electron-dense rims and electron-lucent central regions when stained with methanolic uranyl acetate-lead citrate, and 70% showed this same characteristic rim-staining pattern after limited reaction (30 minutes) for peroxidase with diaminobenzidine. These distinctively large, rim-stained granules were identified in intact, mature peripheral blood neutrophils as well as in human bone marrow promyelocytes, indicating that their synthesis occurs during early myeloid development. This unusual granule type may play a specialized role in the microbicidal functions of the neutrophil, distinct from that of typical azurophil granules.

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HISTORICALLY, human neutrophil granules have been resolved into two major populations; myeloperoxidase-containing azurophil or primary granules and myeloperoxidase-deficient specific or secondary granules.¹ However, evidence is accumulating that a two-granule model is not sufficient to explain a wide variety of experimental observations derived from ultrastructural, subcellular fractionation, and granule secretion studies.²⁻⁶ Recently, Rice et al³ developed a novel Percoll density-gradient system that separated 13 granule fractions from human neutrophils. Peroxidase-reactive granules, shown to be heterogeneous with respect to size and density, were associated with both typical specific and azurophil granule fractions. In addition, a population of myeloperoxidase-containing microgranules⁷⁻⁹ that exhibited physical, morphologic, cytochemical, and secretory properties distinct from the larger and denser peroxidase-reactive azurophil granules was identified among the lowest density fractions.¹⁰

Ganz and co-workers have recently identified several natural antibiotic peptides, termed defensins, which are abundant constituents of human neutrophil and rabbit heterophil granules.¹¹⁻¹⁴ Defensins possessed *in vitro* microbicidal activity toward certain bacteria, fungi, and viruses,^{11,14-16} and ultrastructural immunochemistry localized these peptides to the azurophil granules¹¹ of human neutrophils. The purpose of the present study was to investigate the distribution of defensins among the different isolated granule density fractions reported by Rice et al³ and to determine if a particular defensin-rich granule subpopulation could be identified among the peroxidase-reactive granules. We now report that one particular fraction, H5, which contains the largest and most dense granules, was especially rich in defensins and had distinctive biochemical and ultrastructural properties.

MATERIALS AND METHODS

All investigations using human material were performed with the approval of the Human Investigations Committee, Emory University.

ty, in accord with an assurance filed with and approved by the Department of Health and Human Services.

Isolation of Granule Subpopulations

Human neutrophil concentrates were obtained from leukapheresed healthy adults after informed consent. Neutrophils were further purified to >97% purity following two cycles of hypotonic lysis of erythrocytes and subsequent centrifugation through lymphocyte separation medium (Litton Bionetics, Kensington, MD) to remove mononuclear cells as described previously.¹⁷ Granule-rich postnuclear supernatants were prepared and high (H) and low (L) density gradients were performed as described previously.³ In brief, the H and L gradients each consisted of multiple-step preformed layers of different densities prepared in 50 mL polycarbonate centrifuge tubes. All gradient solutions contained 5 U heparin/mL and were made isotonic by a 1:10 dilution with 10 times concentrated Dulbecco's phosphate-buffered saline (PBS) and adjusted to pH 7.4 with 6N HCl. Appropriate volumes of the resulting 90% Percoll solution (refractive index = 1.3518) were diluted with different volumes of PBS to construct the different density layers. The top layer of each gradient consisted of a maximum of 2.5 mL of postnuclear supernatant from 2.5×10^8 cells. Centrifugation was carried out in a

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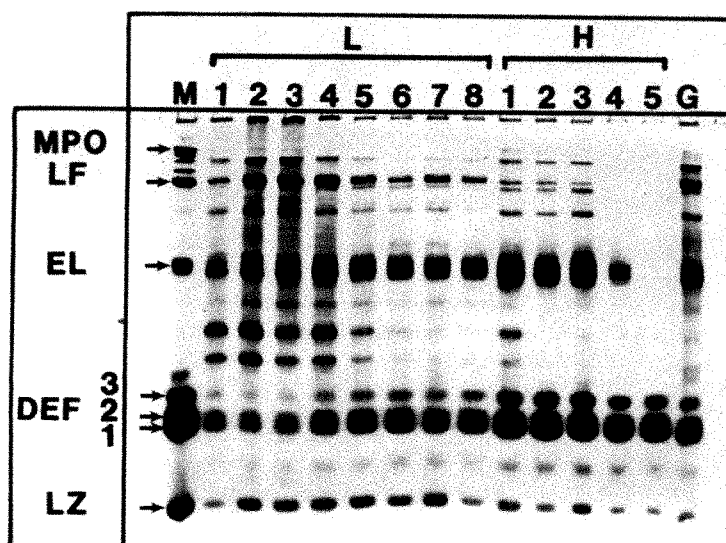


Fig 1. Acid-urea polyacrylamide gel electrophoresis of granule fraction extracts. Lane M contains $\sim 2 \mu\text{g}$ each of purified granule proteins: myeloperoxidase (MPO), lactoferrin (LF), human neutrophil elastase (EL), the three defensins (DEF 1 through 3), and lysozyme (LZ). Lanes L1 through L8 and H1 through H5 each contain $10 \mu\text{g}$ protein from the acid extract of the respective granule fraction. Lane G contains $10 \mu\text{g}$ of the acid extract of unfractionated granules.

Beckman J2-21M centrifuge at 4°C using a JA-20 rotor (34° angle) for 10 minutes at 39,200 g. Gradients were fractionated into 75 samples (0.5 mL) as described previously.³ Appropriate gradient fractions were pooled and centrifuged for 3 hours at 100,000 g at 4°C in a Beckman SW 28 rotor. Granule pellets were collected, washed with PBS, and repelleted by centrifugation at 27,200 g for 15 minutes at 4°C in a Sorvall SM-24 rotor.

Extraction of Granule Proteins and Biochemical Procedures

Granule pellets to be assayed for myeloperoxidase were homogenized in 0.3% cetyltrimethylammonium bromide (or in 0.1% Triton X-100) in PBS, pH 7.4, containing $10 \mu\text{g}/\text{mL}$ of both phenylmethylsulfonyl fluoride and 1-L-tosylamide-2-phenylethylchloromethyl ketone and 1 mmol/L EDTA. Granule lysates were assayed spectrophotometrically for myeloperoxidase using guaiacol as the electron donor and the mass amounts of myeloperoxidase were quantified according to the procedure of Schultz.¹⁸ Myeloperoxidase was also quantified by difference spectroscopy. Granules were homogenized in 0.1% Triton X-100, and absorption spectra from 400 to 600 nm

were measured on reduced (dithionite) minus oxidized samples using an Aminco DW-2a spectrophotometer (American Instrument, Silver Spring, MD). An absorbance coefficient of $75 \text{ mmol/L}^{-1} \text{ cm}^{-1}$ was used for quantifying the characteristic 472-nm peak.¹⁹ Activities of β -glucuronidase were measured as described previously,²⁰ as were lysozyme²¹ and elastase.²² Protein was measured according to Lowry et al²³ using bovine serum albumin (BSA) as a standard except for defensin studies, in which a microbiuret procedure was used.²⁴ For the assay of defensin peptides, granule pellets were extracted in 10% acetic acid, which extracts 80% to 90% of defensins from mixed granules. Defensins were quantified using a recently described enzyme immunoassay²⁵ which used the IgG fraction of rabbit antibody raised against the mixture of defensins 1 through 3.¹¹ The insoluble granule material was taken up into NaOH for micro biuret assay of its protein content. Gel electrophoresis was performed using two different systems: a 12.5% acid-urea polyacrylamide gel and a 10% to 30% gradient sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) system.¹¹ Proteins were visualized by staining with formalin-Coomassie brilliant blue.¹¹ The significance of differences between means of various groups was evaluated using Student's *t* test for unpaired data.

Electronmicroscopy

Granule pellets were overlaid with three drops of human plasma and three drops of 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.35. After 10 minutes at 25°C , the granule-containing plasma plug was minced and submersed in fresh glutaraldehyde-cacodylate buffer for an additional 50 minutes. The minced plug was then washed and stored in 0.1 mol/L cacodylate buffer containing 7% sucrose.

After informed consent from healthy volunteers, either normal bone marrow from the posterior iliac crest was aspirated into a heparinized syringe or peripheral blood was collected by routine venipuncture. Marrow and blood samples were fixed in 0.1 mol/L cacodylate buffer, pH 7.35, containing 3% glutaraldehyde and then rinsed in 0.1 mol/L cacodylate, pH 7.35, containing 7% sucrose as described previously.²⁶

Samples for peroxidase staining were incubated 30 minutes in substrate medium consisting of 3 or 5 mg diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical) in 10 mL 0.05 mol/L Tris-HCl buffer, pH 7.6, to which $33 \mu\text{L}$ of 3% H_2O_2 were added immediately before use as described previously.²⁷ Control samples were incubated in medium lacking H_2O_2 . Staining for sulfate was accomplished using Spicer's high iron diamine (HID) method as

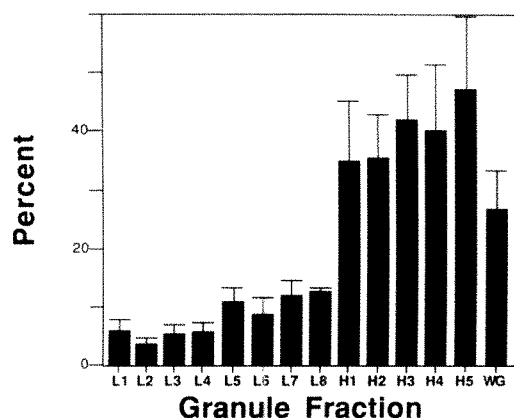


Fig 2. Defensin content of individual granule fractions. Defensin content was determined by enzyme immunoassay and is expressed as a percentage of microgram of defensins per microgram of total protein in each fraction (the different fractions all contain similar amounts of total protein). Values are means from three separate experiments, and bars indicate SEM. WG refers to unfractionated granules.

Table 1. Quantification of Defensins and Typical Primary Granule Markers in High-Density Granule Fractions

Granule Fraction	MPO (6)	β GU (9)	LZ (4)	EL (3)	DEF (3)
H1	72.4 $\pm$ 3.7*	4.7 $\pm$ 0.8*	25.6 $\pm$ 2.8*	0.30 $\pm$ 0.06*	349 $\pm$ 101
H2	53.4 $\pm$ 6.4*	4.8 $\pm$ 0.8*	24.5 $\pm$ 4.2*	0.36 $\pm$ 0.06*	355 $\pm$ 74
H3	61.7 $\pm$ 7.8*	4.1 $\pm$ 0.7	21.8 $\pm$ 2.6*	0.26 $\pm$ 0.01*	422 $\pm$ 74
H4	46.5 $\pm$ 4.2*	4.1 $\pm$ 0.4*	23.4 $\pm$ 6.4	0.20 $\pm$ 0.04*	403 $\pm$ 111
H5	34.6 $\pm$ 3.3	2.8 $\pm$ 0.4	13.2 $\pm$ 3.1	0.10 $\pm$ 0.02	472 $\pm$ 123

Mass amounts of protein, myeloperoxidase (MPO), lysozyme (LZ), and defensins (DEF), and enzymatic activities of β -glucuronidase (β GU) and elastase (EL) were measured in fractions H1 through H5, as described in the Materials and Methods section. Values represent mean protein content or specific activity $\pm$ SE derived from the number of different experiments indicated in parentheses. The values for MPO are expressed as microgram per milligram of protein and β GU as units per milligram of protein, where 1 U equals the amount of enzyme required to hydrolyze 1 nmol of substrate in 1 minute; LZ as micrograms per milligram of protein; EL as nkat per milligram of protein, where 1 nkat equals the amount of enzyme required to hydrolyze 1 nmol of substrate in 1 second and defensins as micrograms per milligram of protein. The values for total protein (μ g $\pm$ SE, $n = 6$) for fractions H1 through H5, respectively, are: 732 $\pm$ 58, 599 $\pm$ 69, 696 $\pm$ 94, 519 $\pm$ 20, and 521 $\pm$ 61.

* $P < .05$ as compared with value for granule fraction H5.

described previously.²⁶ Bone marrow and blood samples processed for staining of vicinal glycol-containing complex carbohydrates were incubated en bloc in α -amylase for removal of glycogen as described previously,²⁸ whereas this step was omitted for isolated cytoplasmic granules. Samples processed for routine morphology and peroxidase cytochemistry were postfixed in 0.1 mol/L cacodylate-buffered 1% OsO₄, whereas those processed for vicinal glycol and sulfate staining were not postfixed. All samples were routinely dehydrated and embedded in Spurr low-viscosity medium. Thin sections of morphologic and peroxidase-stained samples were collected on copper grids. Morphologic specimens were examined after counterstaining with 5% (wt/vol) uranyl acetate (UA) in methanol and 0.4% (wt/vol) lead citrate (LC) in 0.1 N NaOH (5 to 7 minutes in each solution). Peroxidase-stained specimens were not counterstained or were only briefly stained with UALC. Some thin sections of morphologic preparations were stained with aqueous 1% uranyl acetate (pH 4.5)

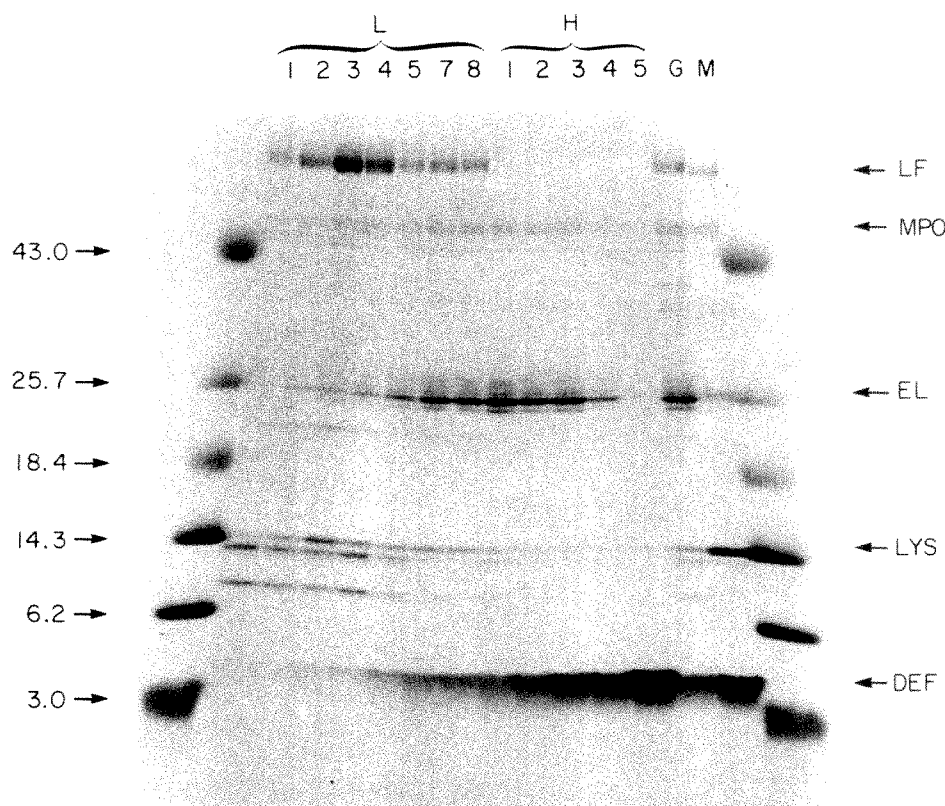
and/or lead citrate, without or with prior tannate treatment to enhance staining of cationic substances.²⁹ Thin sections of specimens processed for vicinal glycol staining were collected on stainless steel grids and stained with a periodate-thiocarbohydrazide-silver proteinate (PA-TCH-SP) method as described previously.³⁰ HID staining was enhanced on thin sections by sequential exposure to TCH-SP as described previously.²⁶ All specimens were examined on a Phillips 301 electron microscope or a Zeiss EM109 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Unusual Composition of High-Density Granule Fraction H5

Human neutrophil granules were separated into 13 isopycnic density fractions using a recently described two-gradient

Fig 3. Ten percent to 30% gradient SDS-PAGE of proteins from density-fractionated neutrophil granules. The two outer lanes contain mol-wt marker proteins whose values are indicated times 10⁻³. Lanes L1 through L8 and H1 through H5 contain 20 μ g acid-extracted protein from each neutrophil granule fraction (fraction 6 was omitted because it did not contain sufficient protein). Lane G contains 20 μ g acid-extracted protein from unfractionated neutrophil granules. Lane M contains purified lactoferrin, LF, 2 μ g; myeloperoxidase, MPO, 4 μ g; elastase, EL, 4 μ g; lysozyme, LYS, 2 μ g; and 2 μ g each of the three defensins (DEF) that migrate together in this gel system. The neutrophil granules used were obtained from a different donor than that in Fig 1.



Percoll centrifugation procedure.³ Acid-extractable proteins were obtained from each of these isolated granule fractions and were electrophoresed into a 12.5% acid-urea polyacrylamide gel (Fig 1). Relative staining intensities of protein bands indicated that fractions L1 through L8 contained most of the specific granules as shown by their high content of lactoferrin. In contrast, fractions H1 through H4 were more enriched with myeloperoxidase and elastase, indicating that they contained most of typical peroxidase-containing azurophil granules. Generally, the intensities of the bands corresponding to defensins (DEF 1 through 3) increased from the lowest density fraction (L1) to the highest density fraction (H5). Based on staining intensity, the five highest density fractions (H1 through H5) were significantly enriched in defensins.

To quantitate this observation, the content of defensins was measured in each of the 13 isolated granule fractions by enzyme immunoassay (Fig 2). Whereas defensins accounted for ~10% of the total protein in each of the low-density fractions (L1 through L8), they comprised between 35% and 50% of the total granule protein in the high-density fractions, H1 through H5.

Defensin concentrations were compared with those of the known azurophil granule components, myeloperoxidase, elastase, lysozyme, and β -glucuronidase (Table 1). Whereas the relative amounts of these azurophil granule components tended to decrease as granule density increased, the converse

was true of defensins. Indeed, defensins were the predominant soluble protein in the H5 granule fraction illustrated in Fig 1. This progressive enrichment in defensins, relative to other soluble granule components as granule density increased, was especially well demonstrated using a gradient SDS-PAGE system (Fig 3). In this case, equal amounts of protein from each fraction were subjected to electrophoresis, although similar results were obtained when equal aliquots of each fraction were used.

Ultrastructural Features of Defensin-Rich Peroxidase-Reactive Granules

Morphology. Morphologic staining using methanolic UALC showed that the granules in fraction H5 were the largest human neutrophil granules. These granules had a mean diameter of $0.32 \pm 0.08 \mu\text{m}$ (mean $\pm$ SD, $n = 200$) as compared with the other typical peroxidase-reactive granule fractions, H1 through H4, which had a combined average diameter of $0.26 \pm 0.07 \mu\text{m}$ ($n = 200$ for each fraction) ($P < .01$).³ Staining with methanolic UALC showed that 70% of the H5 granules ($n = 200$) had an electron-dense peripheral area and an electron-lucent globular central region (Fig 4). This morphologic staining pattern was quite distinctive, since granules in the more typical peroxidase-reactive fractions demonstrated a more homogenous, dense staining pattern (see below). This rim-staining pattern of H5 granules was not evident in specimens stained with lead alone

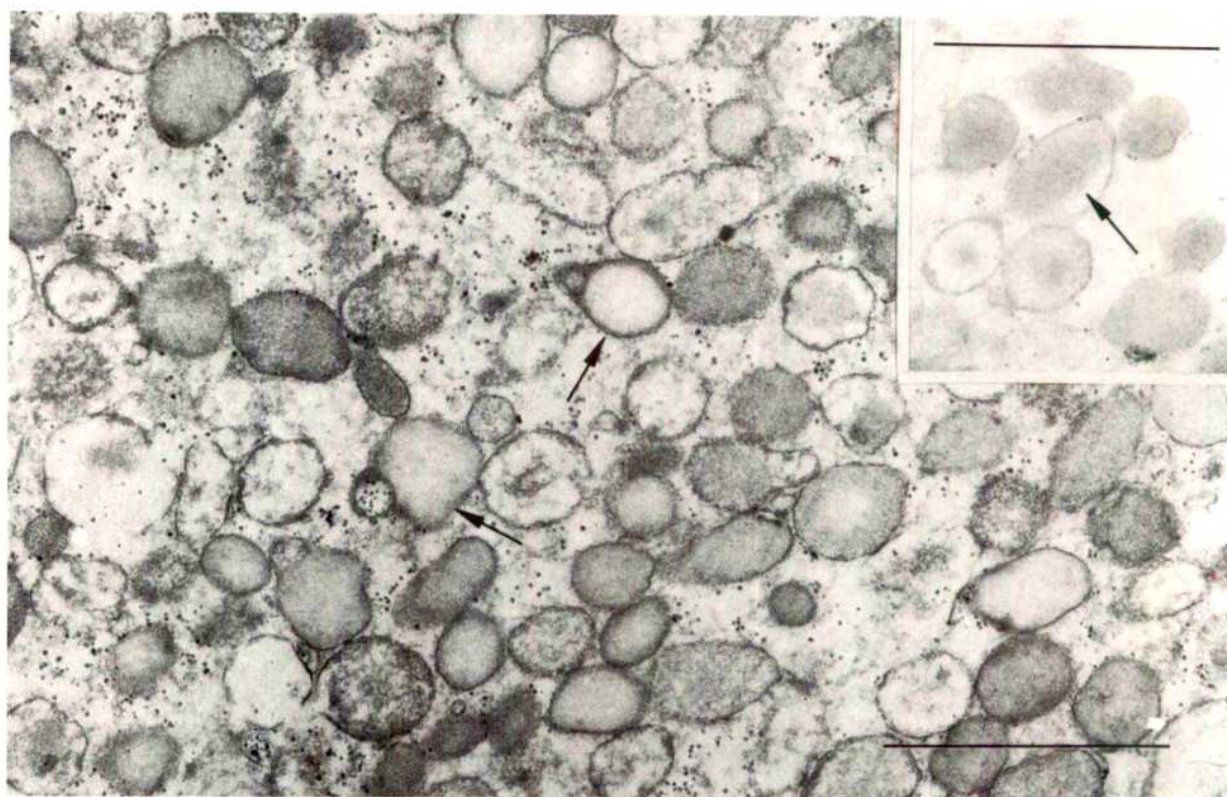


Fig 4. An electron micrograph of granules from fraction H5 after methanolic UALC staining for morphology. About 70% of the granules demonstrated increased rim density (arrows) as compared with the less dense central area. The dense particles between granules represent Percoll. Inset: tannate-lead citrate staining for cationic substances²⁹ failed to accentuate the rim density noted with methanolic uranyl acetate; rather, central areas of the granules appeared dense (arrow). Bars = $1 \mu\text{m}$.

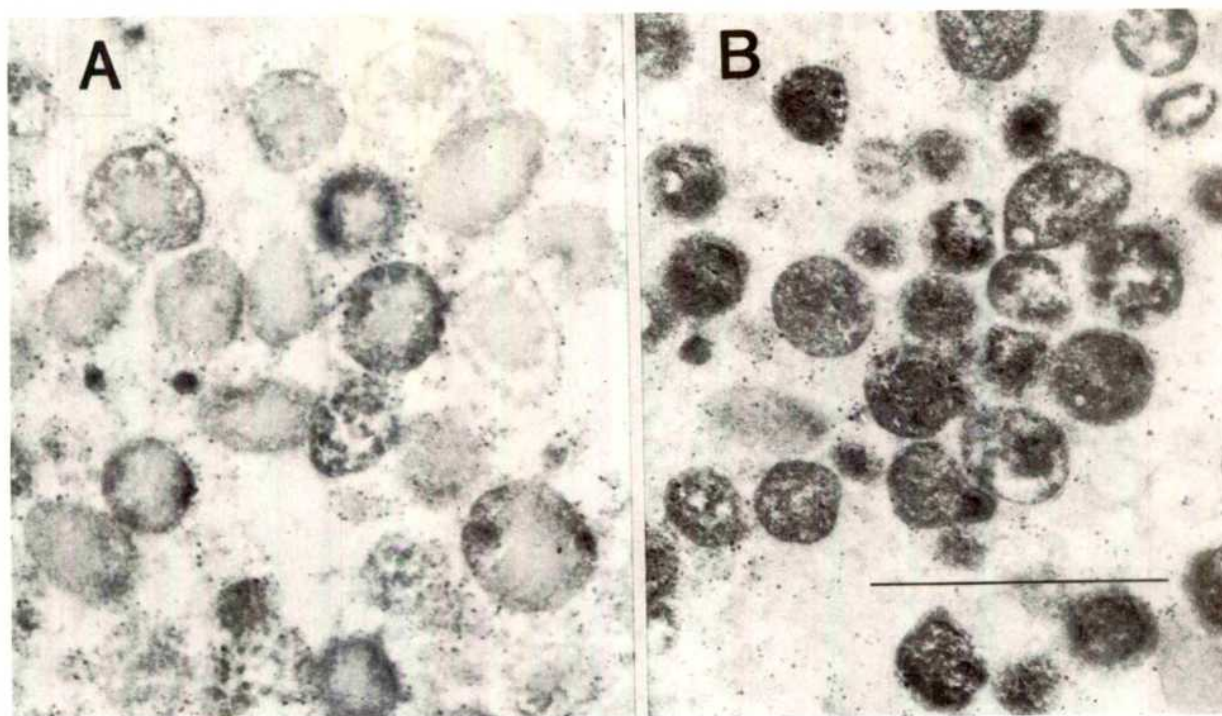


Fig 5. Electron micrographs of granules from fractions H5 and H3 after reaction with DAB for peroxidase activity. Approximately 70% of the granules in fraction H5 (panel A) demonstrated a peripheral (rim) staining pattern, whereas most of the granules in fraction H3 (panel B) exhibited more uniform and intense reactivity. Thin sections were not counterstained with uranyl acetate or lead citrate. Bar = 1 μ m (panels A and B).

or with aqueous uranyl acetate, or with a tannate-lead citrate²⁹ sequence (a procedure that stains cationic proteins) that appeared to result in relatively more central staining than was observed with methanolic UALC (Fig 4, insert).

Peroxidase. Staining for peroxidase with DAB was carried out to determine if the predominant granule morphology type identified in fraction H5 had a reactivity consistent with the quantitative biochemical analyses. As shown in Fig 5A, these granules demonstrated peripheral peroxidase activity and essentially negative reactivity in the central region; no staining was observed in granules incubated in DAB without H_2O_2 . This rim-staining pattern was analogous to that which occurred with the methanolic UALC morphologic staining but was different from the more homogenous DAB staining pattern that occurred in the more typical peroxidase-reactive granules of fraction H3 (Fig 5B). This weak ultrastructural peroxidase reactivity of fraction H5 granules was in agreement with the relatively low level of myeloperoxidase measured biochemically in this granule fraction (Table 1).

The number of granules exhibiting the peroxidatic rim-staining pattern was quantitated independently by two different observers in each of the five high-density fractions. These data, shown in Fig 6, indicated that fraction H5 was enriched about sevenfold in the rim-stained type of granule as compared with fraction H1. Furthermore, the percentage of DAB rim staining granules in fraction H5 (70%) was in good agreement with the percentage (70%) of the granules showing a rim-staining electron density with the UALC morphologic procedure. The remaining 30% of granules in the H5 fraction consisted of large granules that lacked DAB

staining, but otherwise resembled H5 granules in their electron density and size (15%), and granules with the more typical homogeneous DAB staining pattern mentioned previously (15%, cf Fig 5B). The finding of rim-stained granules in fractions other than H5 suggests that density heterogeneity exists even among this granule subclass.

Complex carbohydrates. The large characteristic granules of fraction H5 exhibited only weak matrix reactivity

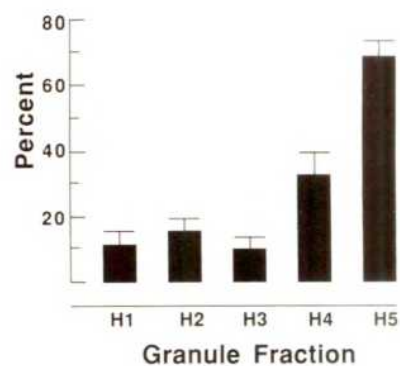


Fig 6. Peroxidatic rim-staining pattern of high-density granules. The percentage of granules having a peroxidatic rim-staining pattern was calculated in each of the five high-density granule fractions following reaction with DAB. Two hundred granules from each fraction were consecutively scored in randomly selected photographs, and each value represents the average of duplicate experiments scored independently by two different observers. Bars indicate range of values. In each experiment, granules were isolated from a different donor.

when stained for vicinal glycol-containing complex carbohydrates using the PA-TCH-SP procedure, similar to that shown previously for granules in intact neutrophils.²⁸ In addition, these same granules stained negatively when reacted for the presence of sulfate-containing macromolecules using an HID procedure.

Intact cells. Methanolic UALC and/or DAB staining of mature peripheral blood neutrophils (Fig 7) showed the presence of rim-stained granules in the intact cell, similar to those observed previously in stained preparations of isolated H5 granules (Figs 4 and 5). If the peroxidase reaction was allowed to continue for 18 hours (rather than 30 minutes) this rim-staining pattern was completely obscured. Similarly, human bone marrow promyelocytes, when stained with methanolic UALC, contained numerous rim-stained granules (Fig 8). The rim staining was of variable intensity and

was obscured by overstaining or omission of methanolic uranyl acetate from the staining procedure. Together, these results confirmed that characteristics of this granule type were not due to artifacts imposed by the fractionation procedure.

DISCUSSION

We recently described a Percoll fractionation procedure that separates human neutrophil granules into 13 fractions by density.³ With this technique, most granules in the eight fractions of lower density (L1 through L8) are peroxidase-negative and most granules in the five fractions of higher density (H1 through H5) are peroxidase-reactive. Examination of the acid-extractable proteins from each of the 13 granule fractions by acid-urea and gradient SDS-PAGE (Figs 1, 3) showed qualitative staining patterns that indi-

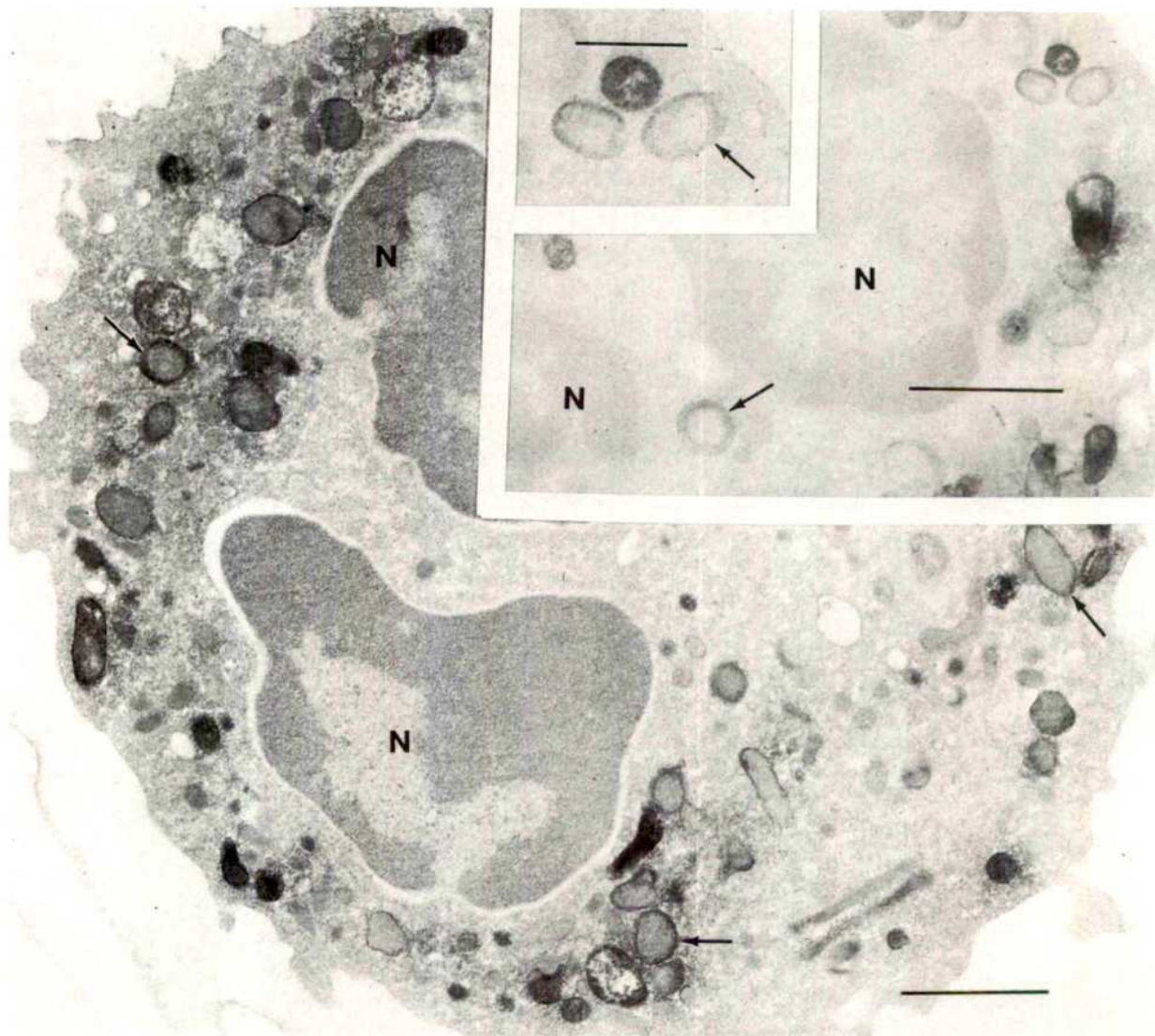


Fig 7. An electron micrograph of an intact peripheral blood segmented neutrophil reacted en bloc with DAB for peroxidase activity and counterstained with methanolic UALC. Numerous granules with increased peroxidase activity in a rim distribution (arrow) are apparent. The larger inset illustrates peroxidase activity in a rim distribution in granules (arrows, enlarged in smaller inset) after DAB incubation, but without methanolic UALC to enhance staining of other organelles. Nucleus (N). Bars = 1 μ m except in smaller inset (0.5 μ m).

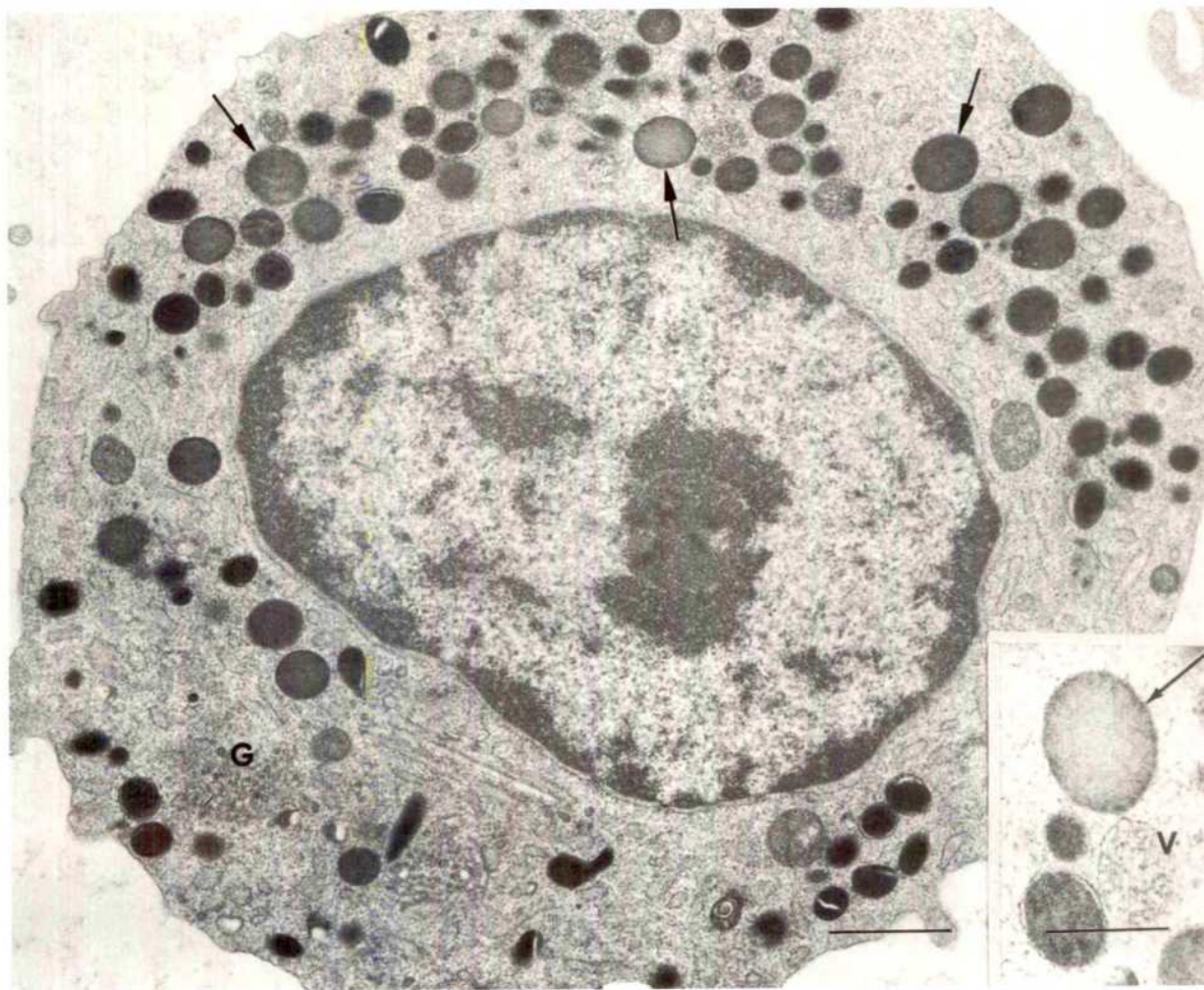


Fig 8. An electron micrograph of a bone marrow promyelocyte stained with methanolic UALC. This cell contains numerous variably electron-dense primary granules. Some granules appear uniformly dense, whereas the larger granules have increased rim density (arrow, enlarged in inset). Condensing vacuole (V), Golgi apparatus (G). Bar = 1 μ m. Inset bar = 0.5 μ m.

cated the higher density granule fractions were enriched in cationic defensins, in agreement with the ultrastructural localization of defensins to large azurophil granules reported previously by Ganz et al.¹¹ Quantification of the defensin content in these granule fractions using a specific enzyme immunoassay demonstrated that the five higher density fractions contained about four times more of these microbicidal peptides than did the eight lower density fractions (Fig 2). In particular, the highest density fraction, H5, was especially rich in defensins (~50% of total protein), but was relatively deficient in the typical azurophil granule marker proteins myeloperoxidase, β -glucuronidase, lysozyme, and elastase when compared with the other four high-density fractions (Table 1).

In addition to their distinctive biochemical and physical properties, the granules of fraction H5 demonstrated distinctive ultrastructural properties. Morphometric analyses showed that they had the largest mean diameter ($0.32 \pm 0.08 \mu$ m) of any human neutrophil granules. Also, when a UALC morphologic stain was used, ~70% of these granules exhibited an electron lucent central area whereas

their periphery was more electron dense (Fig 4). As anticipated, many of these granules stained positively for peroxidase using DAB. Under conditions of limited DAB exposure (30 minutes of incubation), 70% of the H5 granules demonstrated only peripheral peroxidase reactivity and negative staining in their central region (Fig 6). This peroxidase rim-staining pattern (Fig 5A), which has been noted previously by other researchers,^{1,31,32} differed dramatically from the more typical azurophil granules, which exhibited more homogenous and intense peroxidase reactivity (Fig 5B). Hence, the biochemical and physical properties, as well as the morphologic and peroxidase ultrastructural features, distinguish these large dense granules from more typical azurophil granules. Granules with these characteristic morphologic and staining properties were observed in intact mature peripheral blood neutrophils (Fig 7), indicating that this granule type was not an artifact of the granule separation procedure. Moreover, the presence of this class of granules in intact bone marrow promyelocytes (Fig 8) indicated that they were synthesized early in myeloid development.

Previous ultrastructural studies by Ganz et al¹¹ demonstrated the localization of defensins to large azurophil granules. For the moment, technical limitations have precluded the ultrafine structural localization of defensins within H5 granules using immunogold procedures. Nevertheless, many considerations point to the presence of defensins in the distinctive granules of fraction H5 and more specifically to the granule's central region. First, the rim-staining pattern of peroxidase reactivity in H5 granules suggests that the contents may be nonuniformly distributed. Second, since defensins constitute approximately one-half of the total protein in H5 granules, their storage may be assumed to require approximately one-third to one-half of the total granule volume. If we consider an H5 granule to be roughly spherical, its volume will be proportional to the cube of its radius, r , and one-third to one-half of its volume can be calculated to be contained within a central "core" whose radius is 0.69–0.80 r . Third, defensins are carbohydrate-free cationic peptides, and the tannate-lead citrate stain for cationic substances²⁹ showed more staining in the central region of these granules (Fig 4, inset). In contrast, the central core of the large H5 granules exhibited only weak matrix reactivity when stained for vicinal glycol-containing carbohydrates by the PA-TCH-SP procedure³⁸ and did not stain for sulfate-containing macromolecules by the HID procedure.²⁶ Both these findings are also consistent with localization of defensins to the central core area, where they might also mask anionic complex carbohydrate staining.

Most granules in the defensin-rich H5 fraction showed only weak cytochemical peroxidase reactivity, and defensins were also prominent in the other fractions (H1 through H4) that were rich in the more typical azurophil granules. Consequently, the defensin-rich dense granules (DRDGs) in

fraction H5 may represent "immature" or incompletely developed azurophil granules. However, immature azurophil granules are more prominent in promyelocytes and demonstrate strong vicinal-glycol and sulfate staining and are strongly peroxidase reactive.^{8,26,28} In marked contrast, DRDGs were more prominent in late neutrophils, exhibited only weak vicinal glycol staining, no sulfate staining, and showed only weak peripheral peroxidase reactivity.

Alternatively, the neutrophils of certain mammals have been reported to contain abundant granules that are *neither* azurophil nor specific granules.^{33,34} In some cases, these granules are believed to contain antimicrobial components that may prove to be defensins or their homologs.³⁵ Other investigators have interpreted these structures to be dense azurophil granules.³⁶ Therefore, human DRDGs may owe primary phylogenetic allegiance to organelles such as these rather than to the more typical lysosomal azurophil granules. The observation that ~15% of the granules in the H5 fraction did not appear to exhibit peroxidase reactivity is also consistent with such an interpretation.

We recently studied a patient with known "specific granule deficiency," a condition that is associated with frequent and severe infections.³⁷ In addition to abnormal specific and azurophil granules,³⁸ the neutrophils of this patient almost completely lacked defensins and the DRDGs described in the present study.³⁹ Additional experimentation will be required to establish the overall significance of defensins and DRDGs in the repertoire of neutrophil functional responses.

ACKNOWLEDGMENT

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Molecular Determinants of Clinical Expression of Hereditary Elliptocytosis and Pyropoikilocytosis

By Theresa Coetzer, Jack Lawler, Josef T. Prchal, and Jiri Palek

The clinical severity of common hereditary elliptocytosis (HE) is highly variable, ranging from an asymptomatic carrier state to a severe hemolytic anemia. To elucidate the molecular basis of this variable clinical expression, we evaluated 56 subjects from 24 HE kindred, who carry α spectrin mutants characterized by a spectrin dimer (SpD) self-association defect related to a structural abnormality of the α I domain of spectrin. Twenty-nine subjects had common HE, 13 subjects have a closely related disorder, hereditary pyropoikilocytosis (HPP), and 14 are asymptomatic carriers. We compared the severity of hemolysis with the following biochemical parameters: (a) spectrin heterodimer self-association, as manifested by the percentage of SpD in the 4°C low ionic strength spectrin extract; (b) spectrin structure, as examined by limited tryptic digestion of spectrin; and (c) spectrin content of the RBC membrane.

HEREDITARY ELLIPTOCYTOSIS (HE) is a group of disorders that is heterogeneous in terms of inheritance, severity of hemolysis, and RBC morphology as well as the underlying molecular defect.^{1,2} Nine clinical variants of common HE have been delineated.² These include an asymptomatic carrier state; nonhemolytic HE; HE with either minimal, sporadic, chronic, or severe hemolysis; and HE with neonatal poikilocytosis and hereditary pyropoikilocytosis (HPP). This clinical heterogeneity of the HE/HPP syn-

Our analysis indicates that the severity of hemolysis may be correlated with quantitative differences in the percentage of SpD in the 4°C spectrin extract, as well as the total spectrin content of the membrane. Thus, HPP subjects, who have the most severe hemolytic anemia, have the highest percentage of SpD as well as a decreased spectrin content. HE subjects and asymptomatic carriers, respectively, have a lower percentage of SpD and a normal spectrin content. Factors influencing these two determinants include functional differences between the individual spectrin mutants, the relative amounts of mutant spectrin present in the cells, the stability of mutant spectrin, and the possibility of a superimposed genetic defect involving spectrin synthesis.

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drome is paralleled by a biochemical heterogeneity, and several different molecular defects have been described in HE and HPP.² The most common defect found in a subpopulation of HE patients and in all HPP patients is the spectrin dimer (SpD) self-association defect,³⁻⁷ in which the abnormality is localized in the α I domain of spectrin, which represents the SpD-SpD contact site.⁸⁻¹⁹ On a functional level, this defect is manifested by an impaired SpD self-association both in solution and in the membrane, resulting in an increased amount of SpD extracted from the membrane under conditions that reflect the SpD-SpT equilibrium present in the RBC membrane *in situ*.²⁰⁻²² On a structural level, the defect is detected by limited tryptic digestion of spectrin, which cleaves the α and β chains into distinct domains: α I through α V and β I through β IV.²³ To date, three major variants of the α I domain of spectrin have been detected in HE and HPP: Sp α I/74, Sp α I/46, and Sp α I/65 (nomenclature according to references^{1,2}).

The clinical severity of these spectrin α I domain defects varies from that of an asymptomatic carrier state to a severe hemolytic disease. In this study, we attempted to elucidate the molecular basis of these heterogeneous clinical phenotypes. We compared the clinical expression of 56 patients from 24 kindred with HE and/or HPP who have defective SpD self-association to the biochemical expression on (a) a functional level as manifested by the percentage of SpD present in the membrane, (b) a structural level by limited tryptic digestion of spectrin, and (c) a quantitative level by estimating the amounts of normal and abnormal spectrin present in the membrane.

MATERIALS AND METHODS

Subjects. Fifty-six patients from 24 unrelated kindred were studied. Twenty-nine subjects have common HE, including one patient with severe homozygous HE; 13 subjects have HPP; and 14 are asymptomatic carriers. Five of the kindred are white (two with HE/HPP occurring in the same family), one subject has a white mother and a Black father, and the remainder are black. The clinical diagnosis of HE was based on the presence of elliptocytes ($\pm 10\%$ to 100%) since infancy, inherited in most cases as an autosomal dominant trait. For HPP patients, the clinical diagnosis was based on

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the characteristic and persistent RBC morphology of striking poikilocytosis and microspherocytosis, a thermal instability of the RBCs, an autosomal recessive mode of inheritance, and the absence of identifiable abnormalities in glycolytic enzymes and hemoglobin (with the exception of one subject who is homozygous for HbC). Five of the 13 HPP patients and 7 of the 29 subjects with HE were previously reported by our laboratory.^{3,4,8-11} Venous blood was collected from patients and normal control donors in tubes containing acid-citrate-dextrose as anticoagulant. The samples were cooled to 4°C and analyzed either immediately or within 3 days. Blood samples not obtained at St Elizabeth's Hospital in Boston were transported to Boston on ice in insulated containers.

Erythrocyte membrane preparation and quantitation of spectrin content. Erythrocyte ghosts were prepared by hypotonic lysis at 4°C of washed RBCs in 3 mmol/L NaPO₄, pH 8, 0.1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor included to minimize proteolysis.²⁴ Membrane protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5.6% polyacrylamide gels and stained with Coomassie blue.²⁵ Protein concentrations were measured by the BioRad protein assay using bovine serum albumin (BSA) as standard. The amount of spectrin on the membrane was expressed as a ratio of spectrin to band 3 and was quantitated by densitometry of the stained gels at 540 nm and integration of the surface area under the spectrin and band 3 peaks. An alternative method of eluting the Coomassie blue stain from the gel bands by 25% pyridine (vol/vol) was also used in some cases.²⁶ The two methods gave comparable results: For six samples, densitometry yielded a mean Sp/band 3 ratio of 0.98 (range 0.93 to 1.04), and elution gave a mean of 0.97 (range 0.90 to 1.06).

Spectrin extraction and quantitation of spectrin dimers and tetramers. Spectrin was extracted from erythrocyte membranes by incubation in a low ionic strength buffer (0.1 mmol/L NaPO₄, pH 8, 0.1 mmol/L EDTA, 0.1 mmol/L DTT) at 4°C for 16 hours. Spectrin species in the extract were analyzed by nondenaturing electrophoresis in 0.3% agarose 2.5% polyacrylamide gels by the method of Liu et al.³

Limited tryptic digestion of spectrin and quantitation of abnormal spectrin. Spectrin extracts were subjected to limited tryptic digestion and analyzed by two-dimensional isoelectric focusing

(IEF)/SDS-PAGE as previously described.¹⁰ In the case of Sp α I/46 variants, the HPP patients do not produce any normal 80,000-dalton peptide under the conditions of digestion used but instead produce abnormal α I peptides of 46,000 and 17,000 daltons not found in control subjects. The amount of abnormal spectrin in the RBC membranes of Sp α I/46 patients may therefore be estimated by quantitating the amount of abnormal α I peptides present. The ratio of the 46,000-dalton peptide to that of the α II domain or the ratio of the 17,000-dalton peptide to the total gel protein was measured by densitometric scanning at 540 nm of the one-dimensional or two-dimensional tryptic digest maps and subsequent integration of the area underneath the peaks, or by eluting the Coomassie blue stain from the gels with 25% pyridine (vol/vol).²⁶ In the case of Sp α I/74, the amount of abnormal spectrin cannot be accurately quantitated by measuring the amount of 74,000-dalton peptide produced since normal control spectrin produces a certain, although much smaller, amount of the 74,000-dalton peptide. For HE Sp α I/65 variants, neither is precise quantitation of the mutant spectrin possible, since the abnormal 65,000-dalton peptide partly overlaps with the α II domain. However, quantitation of the ratio of these two peptides to the 80,000-dalton peptide is used for comparative purposes in Table 3.

Stability of membrane skeletons. Erythrocyte membrane skeletons were prepared in Triton X-100 and subjected to shear stress in a concentric cylinder-rod shearing apparatus as described by Liu and Palek.²⁷

RESULTS

Clinical characterization. Clinical data on 56 patients from 24 unrelated kindred are shown in Table 1. All subjects have an increased amount of SpD in the membrane and a structural abnormality of the α I domain of spectrin, except in the case of two patients with mild HE and two asymptomatic carriers in whom the structural abnormality of spectrin has not yet been identified (referred to as SpD-SpD?). Classification of the clinical presentation is based on that of Palek² and includes the following clinical variants: asymptomatic carriers, mild HE with no or minimal hemolysis, HE

Table 1. Clinical Data for 56 Patients From 24 Unrelated Kindreds

Clinical Phenotype	RBC Morphology	Subjects (n)	Kindred (n)	Hb (g/dL)	Hct (%)	MCV (fL)	Reticulocytes (%)	Molecular Defect
Asymptomatic carrier	Normal (<2% elliptocytes)	14	11	N	N	N	N	6 Sp α I/74 5 Sp α I/46 1 Sp α I/65 2 SpD-SpD?
Mild HE, no/minimal hemolysis	$\pm$ 10% to 100% elliptocytes	20	12	N	N	N	0.2-4	12 Sp α I/74 6 Sp α I/65 2 SpD-SpD?
Mild HE, chronic hemolysis	15% to 60% elliptocytes	3	1	12-13	35-36	56-72	18-23	3 Sp α I/74
Severe Homozygous HE*	Elliptocytes, poikilocytes, microspherocytes, fragments	1	1	7	20	81	30	1 Sp α I/74
HE with neonatal poikilocytosis	Elliptocytes, poikilocytes, fragments $\rightarrow$ 50% to 100% elliptocytes	5	4	5-11	18-33	50-84	0.3-7	2 Sp α I/74 2 Sp α I/46 1 Sp α I/65
HPP	Poikilocytes, microspherocytes, fragments, few, if any, elliptocytes	13	12	Presplenectomy				7 Sp α I/74
				4-8	13-26	25-72	9-40	5 Sp α I/46
				Postsplenectomy				1 Sp α I/74,
				10-14	24-37	44-70	3-10	46

Abbreviations: Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; N, normal value; SpD-SpD?, subjects with increased percentage SpD but an unidentified structural defect.

*Subject is transfusion dependent, and clinical values were obtained 20 days after transfusion.

with chronic hemolysis, HE with neonatal poikilocytosis, and severe homozygous HE and HPP.

Table 1 indicates that each clinical phenotype does not correspond to a specific structural defect of α spectrin, but that each defect occurs in more than one phenotype. The α I/74 and α I/46 spectrin variants are found in both HE as well as HPP subjects and asymptomatic carriers. The most recently described spectrin variant, Sp α I/65, has thus far only been detected in HE patients and in an asymptomatic carrier, possibly indicating that this structural variant is not as severe as the other two types.

Spectrin dimer content of the membrane and clinical severity. When spectrin is extracted from the RBC membrane at 4°C, the equilibrium between spectrin dimers (SpD), tetramers (pT), and oligomers is stabilized, and the relative amounts of the spectrin species obtained reflect the composition of the membrane in situ.²⁰⁻²² Figure 1 depicts a correlation among disease severity, as reflected by percentage of reticulocytes and hematocrit and the amount of SpD present in RBC membranes of control, HPP and HE subjects, and asymptomatic carriers. The SpD content is expressed as the percentage of SpD of the total SpD and SpT

pool in a crude 4°C spectrin extract. In control subjects, spectrin exists mainly in the tetrameric form and the percentage of SpD is very low (large rectangles in Fig 1, $5.0 \pm 3.6\%$, $n = 35$). In contrast, HPP and HE subjects included in this study, as well as asymptomatic carriers, have an increased amount of SpD in the membrane, reflecting a SpD-SpD self-association defect. This defect is most pronounced in all HPP patients (circles in Fig 1) who have a severe hemolytic anemia (reticulocytes $>10\%$, hematocrit <28), followed by HE patients (rectangles in Fig 1) most of whom have a milder disorder (reticulocytes $\leq 5\%$, hematocrit ≥ 28), and then by asymptomatic carriers (triangles in Fig 1). The differences in the percentage of dimers among patients with low, intermediate, and high reticulocyte and hematocrit values are highly statistically significant (Fig 1). These results indicate that the percentage of SpD in the membrane correlates with the severity of the hemolytic disease and that patients with the highest proportion of SpD in the membrane are the most severely affected. This variation in the levels of SpD is due both to differences in the functional properties of the various mutant spectrins²⁸ (manuscript in preparation) and to differences in the quantity of abnormal spectrin present (see below).

The patient with homozygous HE is not included in the statistical analysis since she is transfusion dependent. She had been transfused 3 weeks prior to our studies, and the low result of 25% SpD in the membrane reflects the SpD content of a mixed population of transfused normal cells and patient cells. However, as an analogy to a similar case report of homozygous HE⁶ and also due to the high levels of SpD found in the membranes of both her parents and sister who are HE heterozygotes (range 38% to 46% SpD), we speculate that she would have a very severe functional and structural defect of spectrin reminiscent of HPP, and that the percentage SpD in her membranes would be markedly increased in accordance with the life-threatening clinical presentation.

Further analysis of the data in Fig 1 shows that there is no significant difference in the percentage of SpD between the Sp α I/74 and Sp α I/46 subjects in each clinical category. However, the structural α I/65 defect differs in functional severity from the α I/74 and α I/46 spectrin variants. HE Sp α I/65 subjects show a significantly smaller increase in the amount of SpD in the membrane ($18.4\% \pm 4.8\%$, $n = 7$) as compared with the HE Sp α I/74 and HE Sp α I/46 variants ($39.4\% \pm 6.3\%$, $n = 19$, $P < .0005$). Furthermore, the asymptomatic carrier of the α I/65 defect has a normal level of SpD in the membrane. Of interest also is one of the asymptomatic carriers of the α I/46 defect who has a reproducibly normal SpD content.

We previously showed that the SpD self-association defect is reflected in a decreased stability of the RBC membrane skeleton when exposed to shear stress.^{2,3,29} Although we find this test useful in screening for membrane skeletal defects, it is inaccurate and poorly reproducible to permit quantitative comparisons.

The relationship between the percentage of SpD in the membrane of the HE patients ($n = 28$) and the percentage of elliptocytes on their peripheral blood smears was also examined. There appears to be no significant correlation between

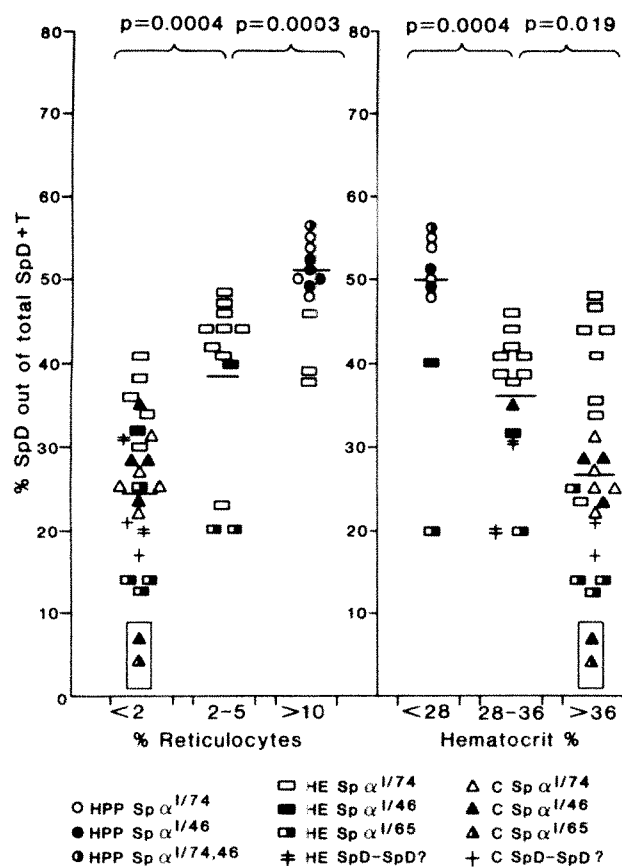


Fig 1. Correlation among the percentage of SpD in the RBC membranes, reticulocytes, and hematocrit of asymptomatic carriers and subjects with HE and HPP. SpD content was quantitated by densitometric scanning. Mean value for each group and the statistical differences in the percentage of SpD among the groups with low, intermediate, and high reticulocyte and hematocrit values is indicated.

Table 2. Correlation of Clinical Phenotype With Amount of Abnormal Sp α I/46

Subject	n	46 kd (%)	17 kd (%)
Control	17	0	0
Carrier Sp α I/46	2	25	23
HE Sp α I/46	1	55	42
HPP Sp α I/46	5	100	100

Percentage of abnormal α I peptides was estimated by densitometric scanning of two-dimensional IEF/SDS-PAGE tryptic peptide maps of spectrin as described in the Materials and Methods section.

the elliptocytic morphology of the RBCs and the self-association state of spectrin in the membrane as indicated by linear regression analysis, which yielded a coefficient of correlation of 0.29 (data not shown).

Quantitation of abnormal spectrin in the cells. The clinical presentation of asymptomatic carrier, HE, and HPP was compared with the approximate percentage of abnormal α I/46 spectrin present, as indicated in Table 2. Control individuals do not produce any of the abnormal 46,000-dalton or 17,000-dalton peptides, and quantitation of these peptides therefore reflects the amount of mutant α spectrin present. Asymptomatic carriers contain $\pm 25\%$ of the α I/46 spectrin, HE patients $\sim 50\%$, and HPP subjects 100% of the abnormal spectrin under the digestion conditions used. A similar approach, however, cannot be used for Sp α I/74 variants since the cleavage site producing the 74,000-dalton peptide is present in the normal α I domain of control spectrin. Accessibility to this site, however, is limited in normal individuals, and only a small amount ($27\% \pm 6\%$) of the 74,000-dalton peptide, relative to the 80,000-dalton peptide, is produced. In contrast, the α I/74 mutant spectrin is characterized by markedly increased susceptibility of this site to cleavage.^{2,8,10}

Total spectrin content of the RBC membrane. The spectrin content of erythrocyte membranes, expressed as a Sp/band 3 ratio, was correlated with clinical expression as illustrated in Fig 2. In control membranes, there are approximately equal amounts of spectrin and band 3 as indicated by a Sp/band 3 ratio of 1.0 ± 0.1 ($n = 60$). In contrast, all 13 HPP subjects show a marked decrease in the Sp/band 3 ratio to a mean value of 0.74 ± 0.05 , reflecting a decreased spectrin content of the membrane. This partial spectrin deficiency is not affected by the type of mutant spectrin present in the cells, since there is no significant difference

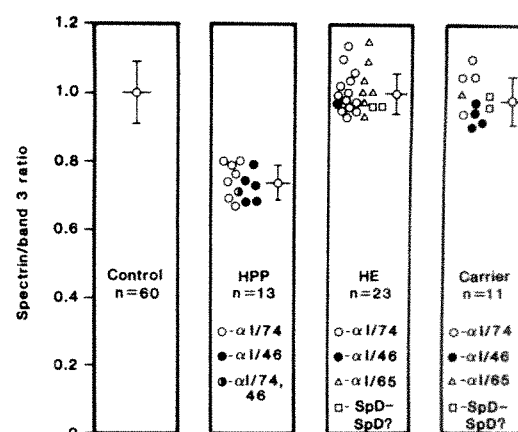


Fig 2. Spectrin content, expressed as a Sp/band 3 ratio, of the RBC membranes of controls, HPP and HE subjects, and asymptomatic carriers. Spectrin was quantitated by densitometric scanning or pyridine elution of Coomassie-blue stained SDS polyacrylamide gels. Mean $\pm$ SD is given for each group.

between the Sp/band 3 ratio of HPP Sp α I/74 (0.75 ± 0.05 , $n = 7$) and HPP Sp α I/46 (0.72 ± 0.05 , $n = 5$) and a value of 0.71 for the double heterozygote HPP Sp α I/74, 46.

HE patients ($n = 23$) have a normal Sp/band 3 ratio of 0.99 ± 0.07 , and asymptomatic carriers also fall into the normal range with a mean Sp/band 3 ratio of 0.98 ± 0.06 ($n = 11$). Thus, even though the functional and structural defects of spectrin in HE and in asymptomatic carriers are qualitatively the same as in HPP subjects, the spectrin content in HE and carriers is not affected.

HE with neonatal poikilocytosis. Five patients in this study exhibited HE with neonatal poikilocytosis. This condition was noted in association with all three structural variants of spectrin as indicated in Table 1. Table 3 depicts an α I/74 and the α I/65 variant in which the change in the RBC morphology is compared with the aboveoutlined biochemical parameters. This type of analysis was not performed on the α I/46 variants. Table 3 indicates that the percentage of SpD in the membrane, as well as the relative amounts of the abnormal α I peptide, remains constant during the transformation from HPP to HE phenotype. The Sp/band 3 ratio also remains within the control range during this transition. These data indicate that the functional and structural defects of spectrin remain unaltered during the morphologic transi-

Table 3. Correlation of RBC Morphology with Biochemical Features of Spectrin in HE With Neonatal Poikilocytosis

Subject	Age	Morphology		SpD (%)	Sp/b3	74 kd* (%)	65 kd† (%)
		Elliptocytes (%)	Poikilo-/Microcytes (%)				
DD (HE Sp α I/74)	2 wk	35	55	47	1.02	49	0
	15 mo	30	20	48	0.96	43	0
TE (HE Sp α I/65)	3 wk	35	55	19	0.99	—	72
	3½ mo	—	—	19	1.09	—	64
	10 mo	65	30	20	1.03	—	76
	16 mo	30	16	21	1.12	—	69

*Densitometric scanning of tryptic digest maps of spectrin was used to estimate 74 kd (%) and is expressed as a percentage of the total 74,000 + 80,000-dalton peptides.

†Percentage 65,000 dalton + β II peptides in the total 65,000 + β II + 80,000-dalton peptides is given as 65 kd (%).

tion, implying that intracellular factors rather than structural changes in the spectrin molecule are responsible for the change in clinical presentation.

DISCUSSION

In recent years, we and other researchers identified four structural variants of α spectrin associated with HE.^{8-12,14} These mutant spectrins also exhibit a functional SpD self-association defect. During these studies, a marked variability became apparent in the clinical expression of the HE disorder, ranging from an asymptomatic carrier state to mild HE with minimal or moderate hemolysis and ultimately to HPP, which presents as a severe hemolytic disease. The purpose of the present study was to elucidate the molecular basis of these heterogeneous clinical phenotypes. To accomplish this objective, we compared the biochemical expression of the underlying molecular defects in 56 HE/HPP subjects from 24 kindred who exhibit a SpD self-association defect with the clinical severity and morphologic expression of the disorder. Our analysis of the functional and structural abnormalities of spectrin in these HE/HPP patients indicates that the heterogeneity of clinical expression may be correlated with two major factors: the SpD content and the total spectrin content of the membrane.

SpD content of the membrane. The role of the SpD self-association defect as a determinant of the clinical severity is illustrated by the highly statistical differences in the percentage of SpD between the low, intermediate, and high reticulocyte and hematocrit groups. In addition, all HPP patients, who have a very severe hemolytic anemia, have the highest amount of SpD in the membrane, whereas HE subjects have a considerably lower percentage of SpD and are clinically much less affected (Fig 1). Furthermore, asymptomatic carriers have an even lower percentage of SpD in the membrane.

The amount of SpD in turn appears to be related to at least two factors. The first factor involves differences between mutant α spectrins in terms of their functional capacity to self-associate into SpT. This possibility is raised by previous studies in our laboratory^{10,28} that demonstrated that the self-association of α I/74 spectrin is severely impaired whereas the α I/46 spectrin variant retains some capacity to form SpT.

The second factor is the amount of dysfunctional α spectrin present. For Sp α I/46 variants, the amount of abnormal α I peptides (46,000 and 17,000 daltons) may be quantitated and corresponds to the amount of abnormal spectrin present, since control spectrin does not produce any of these peptides. The clinical severity correlates with the mutant spectrin content, since HPP Sp α I/46 individuals have no normal α I domain (ie, 100% abnormal spectrin), followed by HE Sp α I/46 subjects and carriers who contain ~50% and 25% of abnormal spectrin, respectively (Table 2). This type of correlation is not possible for the α I/74 and α I/65 spectrin variants, since the amount of abnormal α I peptides cannot be accurately quantitated, as previously discussed.

Total spectrin content of the membrane. The clinical expression also appears to be related to the net spectrin content of the membrane. HPP RBCs contain ~30% less

spectrin than do those of control individuals, whereas all HE patients, most of whom have a much milder form of the disease, as well as asymptomatic carriers, have a normal spectrin content (Fig 2). However, the estimation of spectrin content from the intensity of staining of SDS-PAGE slabs does not accurately quantitate the actual degree of spectrin deficiency, which is likely to be even larger.³⁸ Because in addition to the partial spectrin deficiency, HPP RBCs also contain the highest fraction of dimeric spectrin, it remains uncertain whether this partial spectrin deficiency in HPP aggravates the other molecular abnormality of spectrin (defective spectrin self-association). However, spectrin deficiency in HPP might be responsible for microspherocytosis in this condition, which is absent in patients with HE. Partial deficiency of spectrin is also a common feature of hereditary spherocytosis³⁸ and (although it remains to be proven) may underlie the decrease in RBC surface area in this condition.

Several factors could influence the spectrin content of the membrane and hence the clinical expression. These include the following: The instability of mutant spectrin leads to degradation of this protein prior to its assembly on the membrane (as may be the case in HPP Sp α I/46.³⁰ Second, a double heterozygous state for an α spectrin mutant and a defect involving synthesis of this protein is suggested by a characteristic pattern of HPP inheritance as previously discussed.² Typically, one of the parents of the HPP offspring carries the α spectrin mutant, whereas the other parent is fully asymptomatic. It is likely that this other parent transmitted to the HPP offspring another genetic defect (presumably involving synthesis of spectrin) that increases the quantitative expression of the α spectrin mutant and causes a concomitant partial spectrin deficiency. A third possible factor accounting for the clinical and molecular heterogeneity of HE could be a duplication of the α spectrin gene. This hypothesis is tentatively supported by the correlation between the amount of abnormal spectrin present in Sp α I/46 subjects and the clinical expression. The Sp α I/46 asymptomatic carriers have $\pm 25\%$, HE subjects $\pm 50\%$ and HPP patients 100% of the abnormal spectrin (Table 2). These results could be compatible with a duplicated α spectrin gene, and consequently, inheritance of one, two, or more abnormal alleles of four spectrin alleles could result in an asymptomatic carrier state, mild HE, and homozygous HE or HPP, respectively. The human α spectrin gene has been localized to chromosome 1³³ and, with the aid of genetic cloning and hybridization techniques as well as studies on kindred carrying several nonlinked variants of α spectrin, the validity of the α spectrin gene duplication hypothesis could be established.

HE with neonatal poikilocytosis. HE with neonatal poikilocytosis is an interesting and heterogeneous phenotypic variant of HE illustrating the influence of additional factors on the variability in clinical expression. During the clinical transition of HE, the severity of the underlying molecular abnormalities does not change (Table 3), and the poikilocytosis and hemolysis in the neonate and the subsequent morphologic transition has been considered as possibly related to differences between the RBC microenvironment of the neonate and the adult.³⁴ The presence of fetal hemoglo-

bin, which has a decreased affinity for 2,3 DPG, would lead to increased levels of 2,3 DPG in the cytoplasm. This could destabilize the spectrin/actin/band 4.1 complex,^{35,36} which could in turn augment the expression of the SpD self-association defect and thus weaken the skeleton even further, leading to poikilocytosis and fragmentation.³⁴ Whether the neonatal poikilocytosis occurs in all neonates with HE, or in a subset of such patients remains to be established. One neonate with presumed HE, in whom the molecular defect has not been defined, did not have neonatal poikilocytosis.³⁴ On the other hand, all HE neonates carrying α spectrin defect who are reported in this study did have neonatal poikilocytosis.

In conclusion, this comparative study indicates that HE subjects, who exhibit a SpD self-association defect, have the same defect of α spectrin as do HPP individuals. The variation in the severity of hemolysis, ranging from asymptomatic carrier to HE and HPP, is related to differences in the SpD content and presumably the total spectrin content of the membrane as well.

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Effect of Warfarin on Prothrombin Synthesis and Secretion in Human HEP G2 Cells

By Simon Karparkin, Thomas H. Finlay, Anita L. Ballesteros, and Margaret Karparkin

Prothrombin synthesis and secretion were studied in a human hepatoma cell line (Hep G2) incubated with ^{35}S -methionine for 2 to 24 hours at 37 °C. Extracellular and intracellular prothrombin were detected by immunoprecipitation with affinity-purified antiprothrombin antibody. Incorporation of ^{35}S -methionine into prothrombin was monitored by counting specific bands excised from 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Prothrombin represented 0.3% to 0.7% of total newly synthesized protein secreted into the media. Warfarin had no effect on total prothrombin synthesis (extracellular plus intracellular). However, warfarin inhibited secretion of newly synthesized prothrombin by 58% to 73% over a 2 to 4 hour period. This was accompanied by the intracellular accumulation of an immunoprecipitable species of prothrombin of 78 kd, 6 kd less than extracellular prothrombin. At the end of 4-hour incubation with warfarin, intracellular prothrombin increased from 44% to

82% (twofold) of total prothrombin, whereas extracellular prothrombin decreased from 56% to 19% (threefold) of total prothrombin. After 24-hour incubation with warfarin, intracellular and extracellular immunoprecipitable prothrombin approached control values. Deglycosylation of extracellular and intracellular prothrombin with hydrofluoric acid (HF) resulted in a decrease in mol wt for both species to 66 kd. Endoglycosidase-H treatment, which digests "early mannosyl" residues, resulted in a decrease in the mol wt of the intracellular species of 8 kd with no effect on the extracellular species. Thus, the lower mol wt intracellular species that accumulates following early warfarin treatment is due to the presence of incompletely processed carbohydrate chain. The data are compatible with the hypothesis that optimum glycosylation and secretion require Vitamin K-dependent carboxylation.

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THE ROLE of warfarin in decreasing plasma prothrombin biologic activity has been known for 41 years.¹ In humans, this is associated with decreased secretion of an inactive molecule, as determined by immunoassay.^{2,3} The inactive molecule has the same approximate mol wt as the biologically active molecule but is missing the carboxylated residues on the γ carbon of ten glutamic acid residues at the N terminal end (first 33 residues) of the molecule.⁴⁻⁶ Vitamin K activates a microsomal carboxylase which carboxylates these ten glutamic acid residues at the γ carbon site.^{7,8}

We studied the effect of warfarin on human Hep G2 cells and found that intracellular prothrombin accumulates following warfarin treatment as a lower mol wt precursor. An ~6,000-kd difference between extracellular and intracellular prothrombin appears to be due to partial glycosylation of the prothrombin molecule due to a block in the removal of mannosyl residues. The details of these investigations are cited below.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical, St Louis, unless indicated otherwise. Minimum essential medium (MEM) was obtained from GIBCO, Grand Island, NY. Warfarin was obtained from Endo Laboratories, Garden City, NY. Sodium dodecyl sulfate (SDS), polyacrylamide, and Affi-gel 15 were obtained from BioRad, Richmond, CA. HF was obtained from Fisher Scientific, Fairlawn, NJ. Endoglycosidase-H was obtained from Boehringer Mannheim, Indianapolis. ^{35}S -Methionine (1,000 Ci/mmol) was obtained from New England Nuclear, Boston. ^{125}I -Staphylococcal protein A (30 mCi/mg) was obtained from Amersham, Arlington Heights, IL.

Human hepatoma G2 cells (obtained from American Type Culture Collection, Rockville, MD) were grown in MEM plus 10% fetal calf serum (FCS) until confluence. They were then washed in methionine-free MEM (GIBCO) and incubated in methionine-free MEM with or without 10% FCS or 1 to 5 $\mu\text{g}/\text{mL}$ warfarin (Endo) in 25-cm² tissue culture flasks (Falcon, Becton Dickinson, Oxnard, CA) for 1 to 24 hours at 37°C with ^{35}S -methionine (150 $\mu\text{Ci}/\text{mL}$) and 10⁻⁴ mmol/L phenylmethylsulfonyl fluoride (PMSF) in a total volume of 3 mL containing ~1 $\times$ 10⁶ cells. Extracellular and intracellular total synthesized protein and prothrombin were deter-

mined after careful attention to experimental detail, which required development of reliable methodology for these measurements.

Total synthesized protein. Total synthesized protein secreted into the medium and present in the cell lysate was determined by applying an aliquot of either material (50 μL of supernatant, 50 μL of Triton X-100 lysate) to 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE)⁹ and counting radioactivity in regions of the gel corresponding to proteins of 10 to 150 kd. This methodology was necessary to obtain reproducible quantitative data. Early experiments in which proteins were analyzed for radioactivity after precipitation with trichloroacetic acid (TCA) proved unreliable because of nonspecific adsorption of radioactivity. Cell lysate was prepared by addition of 3 mL 0.5% Triton X-100 to the tissue culture flask, which had been aspirated of media and washed three times in 3 mL fresh media. The suspension was boiled in 2% SDS for 2 minutes, and the 100,000 g supernatant was used for assays.

Prothrombin synthesis. Prothrombin synthesis was determined by immunoprecipitation of medium or cell lysate (boiled in 2% SDS) with affinity-purified rabbit antiprothrombin antibody. The complex was isolated by addition of 200 μL media or cell lysate to 600 μL buffer A plus 40 μL affinity-purified antiprothrombin antibody (0.2 mg/mL) and was incubated overnight at 4°C. Buffer A contained 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 0.02% sodium azide, and 50 mmol/L Tris-HCl, pH 7.4. Eighty microliters of a 50% suspension of insoluble staphylococcal cell wall protein A (Sigma) in buffer A was then added, and the mixture was incubated at room temperature for 2 hours. The suspension was centrifuged at 13,000 g, washed six times in buffer A, and resuspended in 50 μL of

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buffer A. An equal volume of a solution containing 2% SDS, 10% glycerol, 5% mercaptoethanol, 10 μ g prothrombin, 0.06 mmol/L Tris-HCl, pH 6.8, was then added, and the suspension was heated at 95°C for 5 minutes. The material was then centrifuged as above, and the supernatant was applied to 10% SDS-PAGE for excision of radioactive bands following staining with Coomassie brilliant blue, as well as for autoradiography (Fig 1). Excision of the specific radioactive prothrombin bands eliminated the error of nonspecific adsorption of contaminating radioactive bands to the immune precipitate. This methodology was necessary to obtain reproducible quantitative data.

Preparation of affinity-purified rabbit anti-human prothrombin antibody. Human prothrombin purified by the method of Bajaj and Mann¹⁰ showed one band on 10% SDS-PAGE prior to immunization. Rabbit antiprothrombin IgG was purified by chromatography on diethylaminoethyl (DEAE)-52 after precipitation from serum with 50% saturated ammonium sulfate. The precipitate was dissolved in 0.1 molar phosphate buffer, pH 7.6, dialyzed against the same buffer and applied to a DEAE-52 column equilibrated in the same buffer. The flowthrough material contained purified IgG. The purified IgG was applied to an Affi-gel 15 column to which 5 mg/mL purified human prothrombin had been coupled. The adsorbed antibody was eluted with 0.1 mmol/L glycine, pH 2.5, and neutralized with 1 mmol/L Tris buffer to a pH of 7.0. This antibody, when used in a Western Blot of human plasma, developed with ¹²⁵I-protein A (0.83 μ Ci/mL) in 0.01 mmol/L phosphate buffer, 0.15 mmol/L NaCl, 0.05% Tween-20, pH 7.4, and washed in the same solution, gave a single band of ~84 kd on autoradiography.

Deglycosylation. Deglycosylation was performed by treatment of aliquots of cellular or media samples with HF by the method of Keutmann et al.¹¹ Prothrombin-containing samples were immunoprecipitated as described above, and the immune complexes were collected on immobilized protein A (Sigma). The prothrombin (along with IgG) was removed from the agarose beads by heating in 1% SDS at 90°C for 10 minutes, followed by centrifugation. The sample was then reduced with 1 mmol/L 2-mercaptoethanol and

alkylated with 1.1 mmol/L iodoacetamide. After dialysis against water, the samples were lyophilized in Teflon-coated flasks and then suspended in 1 mL anisole. After evacuation, samples were cooled in liquid nitrogen, and 10 mL of HF (condensed by cooling in liquid nitrogen) was added. After vigorous stirring at 0°C for 1.5 hours, the HF was removed under vacuum. Residual anisole was removed by washing with cold anhydrous ether, and the deglycosylated proteins were dialyzed against phosphate-buffered saline (PBS) prior to SDS-PAGE.

Endoglycosidase-H digestion. Endoglycosidase-H digestion was performed with aliquots of cellular or media samples to determine the presence or absence of "high" mannose oligosaccharide chains by a modification of the method of Tarentino et al.¹² Prothrombin-antibody complexes were collected on immobilized protein A as described above. The beads were then washed in 0.01 mmol/L PBS, pH 7.4, suspended in PBS containing 2 mmol/L PMSF and incubated with 20 mU/mL of endoglycosidase-H overnight at 30°C. Residual prothrombin was eluted with 1% SDS and heated at 90°C as above, and the supernatant was applied to SDS-PAGE.

RESULTS

Synthesis of a lower molecular weight intracellular prothrombin species and effect of cycloheximide and warfarin on newly synthesized extracellular and intracellular prothrombin. Figure 2 shows an autoradiogram of prothrombin synthesis at 2 and 4 hours of incubation without and with cycloheximide (10^{-5} mmol/L) or warfarin (1 μ g/mL). The extracellular apparent 84-kd band (± 2.3 , SD, nine experiments) immunoprecipitated by antiprothrombin antibody in lanes 1 and 4, representing newly synthesized prothrombin, and the apparent 78-kd band (± 4.5 SD, nine experiments) in lanes 7 and 9, representing newly synthesized intracellular prothrombin. Cycloheximide inhibited extracellular (lanes 2

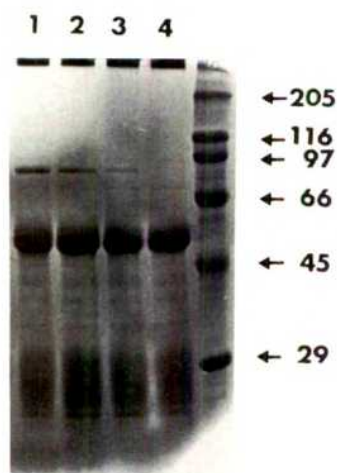


Fig 1. Coomassie brilliant blue stain of 10% SDS-PAGE of an aliquot of media obtained from a 4-hour incubation with 1×10^6 Hep G2 cells. Varying concentrations of purified human prothrombin were added after immunoprecipitation with affinity-purified rabbit anti-human prothrombin antibody. Lanes 1 through 4 refer to addition of prothrombin at 25, 12.5, 6.25, and 0 μ g. The gel was dried on Whatman 3 MM paper, and the region corresponding to the prothrombin band was excised for radioactive measurements of prothrombin synthesis in other studies. The position of standard mol wt markers is shown (far right lane) in kilodaltons.



Fig 2. Autoradiogram after 10% SDS-PAGE of immunoprecipitates of aliquots of media and Hep G2 cells incubated with cycloheximide or warfarin for 2 and 4 hours at 37°C in MEM plus ³⁵S-methionine. Lanes 1 through 3 represent aliquots of media from control, cycloheximide (2×10^{-5} mmol/L) or warfarin (1 μ g/mL) incubations at 2 hours. Lanes 4 through 6 represent the same aliquots at 4 hours. Lanes 7 through 9 represent aliquots of cell lysate from control, cycloheximide, or warfarin-treated cells at 4 hours. Outer lanes refer to positions of radioactive standard mol wt markers.

and 5) and intracellular (lane 8) prothrombin synthesis. Warfarin inhibited the secretion of newly synthesized prothrombin (lanes 3 and 6) but resulted in the accumulation of newly synthesized intracellular prothrombin at 4 hours (lane 9). Confirmation that the radioactive bands at 84 and 78 kd were prothrombin was obtained by demonstrating inhibition of immunoprecipitation with purified human prothrombin at 1 mg/mL (Fig 3) with lack of inhibition at 100 μ g/mL prothrombin (data not shown).

Effect of deglycosylation on extracellular and intracellular prothrombin species. Figure 4 shows the effect of deglycosylation on the mol wt of intracellular and extracellular prothrombin without and with warfarin. The resultant identical apparent mol-wt species is ~66 kd.

Effect of endoglycosidase-H on extracellular and intracellular prothrombin species. Figure 5 shows the effect of endoglycosidase-H on the molecular weight of intracellular and extracellular prothrombin without and with warfarin. The decrease in apparent mol wt of the intracellular species at 2 and 4 hours from 79 (lanes 6 and 8, respectively) to 71 kd (lanes 5 and 7, respectively), without an effect on the extracellular mol-wt species (lanes 1 through 4), indicates the presence of mannosyl residues on the intracellular species.

Cycloheximide inhibition of total secreted protein synthesis. Figure 6 shows a kinetic analysis of secreted newly synthesized total protein without and with cycloheximide, at 2 and 4 hours. Cycloheximide inhibited secreted total protein synthesis by ~75%.

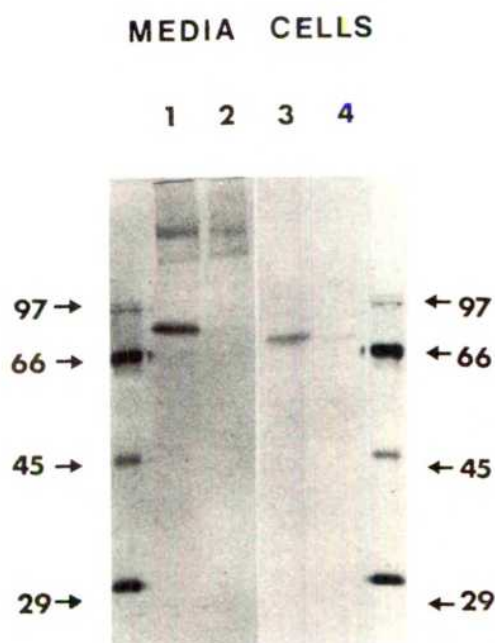


Fig 3. Autoradiogram after 10% SDS-PAGE of radioactive media and cellular prothrombin bands immunoprecipitated without and with prothrombin. Lanes 1 and 2 represent aliquots of media immunoprecipitated without and with prothrombin 1 mg/mL. Lanes 3 and 4 represent aliquots of cell lysate immunoprecipitated without and with prothrombin 1 mg/mL. Outer lanes refer to position of radioactive standard mol wt markers.

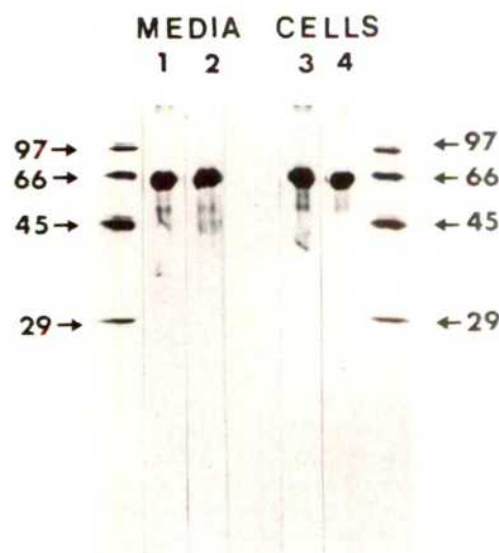


Fig 4. Effect of deglycosylation on mol wt of extracellular and intracellular prothrombin bands. Immunoprecipitated ^{35} S-labeled prothrombin samples were eluted with SDS and heated, alkylated, suspended in anisole, and deglycosylated with HF (described in the Materials and Methods section). Labeled proteins were visualized by autoradiography after 10% SDS-PAGE. Lanes 1 and 3 represent deglycosylated aliquots of media and cells, respectively, following 2-hour incubation. Lanes 2 and 4 represent similar aliquots following 4-hour incubation. Outer lanes refer to positions of radioactive standard mol wt markers.

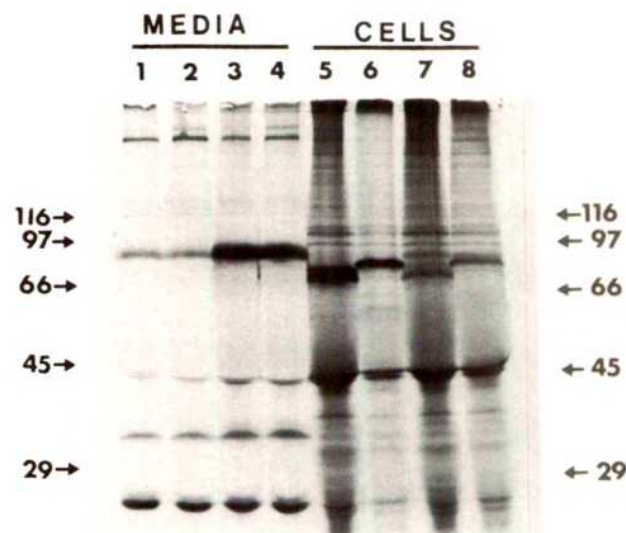


Fig 5. Effect of endoglycosidase-H on mol wt of extracellular and intracellular prothrombin bands. Immunoprecipitated samples were isolated on immobilized protein A and treated with endoglycosidase-H, 20 mU/mL overnight at 30°C. Residual prothrombin was eluted (described in the Materials and Methods section) and applied to 10% SDS-PAGE. Labeled proteins were visualized by autoradiography. Lanes 1 and 2 represent aliquots of media with and without endoglycosidase-H treatment following 2-hour incubation. Lanes 3 and 4 represent similar aliquots following 4-hour incubation. Lanes 5 and 6 represent aliquots of warfarin-treated cell lysates with and without endoglycosidase-H treatment following 2-hour incubation. Lanes 7 and 8 represent similar aliquots following 4-hour incubation. Outer lanes refer to positions of standard mol wt markers by Coomassie brilliant blue staining.

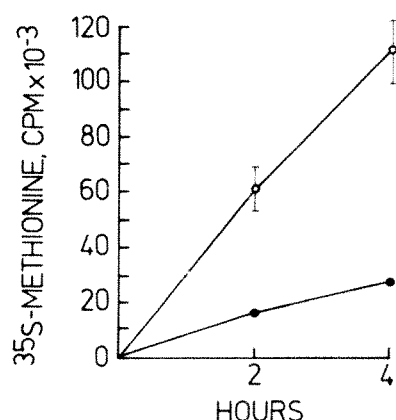


Fig 6. Effect of cycloheximide on newly synthesized total secreted protein. Hep G-2 cells (1×10^6) were incubated without (O—O) 16 experiments, and with (●—●) two experiments of 2×10^{-5} mmol/L cycloheximide. Aliquots were removed at 2 and 4 hours, boiled in 2% SDS, and subjected to 10% SDS-PAGE. The bands representing mol wt of 10 to 150 kd were excised. Radioactivity was assayed in a scintillation counter.

Effect of warfarin on newly synthesized secreted prothrombin. Newly synthesized secreted prothrombin varied between 0.27% and 0.67% of total secreted protein synthesis during a 2- to 24-hour period of incubation (Table 1). Warfarin inhibited secretion of newly synthesized immunologically detectable prothrombin by 58% to 73% during a 2-to 4-hour period. However, this inhibition was no longer detectable after 24 hours of incubation (Fig 7), media lane 2 (4 hours) as compared with lane 4 (24 hours). This could be accounted for by a decrease in warfarin-induced accumulation of intracellular prothrombin at 24 hours as compared with accumulation at 4 hours (Fig 7, cells in lane 4 (24 hours) as compared with lane 2 (4 hours)). A kinetic analysis is cited below.

Effect of warfarin on extracellular and intracellular newly synthesized prothrombin at 4 and 24 hours. In the experiment to determine the effect of warfarin on extracellular and intracellular newly synthesized prothrombin at 4 and 24 hours, prothrombin synthesis at 4 hours represented $0.39\% \pm 0.04\%$ (SEM) of total protein synthesis: 56% was extracellular and 44% intracellular. Warfarin (1 $\mu\text{g/mL}$) had no effect on total prothrombin synthesis, (442 ± 43 (SEM) v 511 ± 56 cpm, $P > .2$, with and without warfarin,

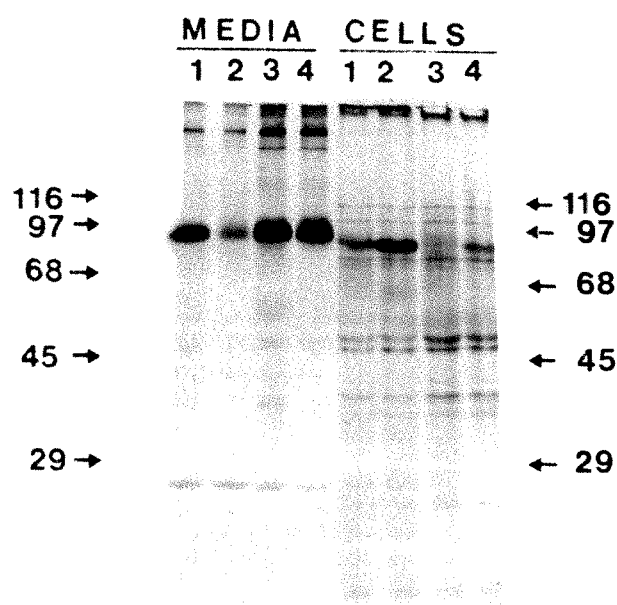


Fig 7. Effect of warfarin on newly synthesized extracellular and intracellular prothrombin at 4 and 24 hours. Labeled prothrombin was visualized by autoradiography after 10% SDS-PAGE of immunoprecipitates. Lanes 1 and 2 represent aliquots of media or cells without and with warfarin (5 $\mu\text{g/mL}$) following 4-hour incubation. Lanes 3 and 4 represent similar aliquots following 24-hour incubation. Outer lanes refer to positions of standard mol wt markers by Coomassie blue staining.

respectively, eight experiments). However, extracellular secretion decreased to 19% (3-fold, $P < .001$), and intracellular accumulation increased to 82% (1.9-fold, $P < .001$, Fig 8).

Prothrombin synthesis at 24 hours represented $0.31\% \pm 0.08\%$ of total protein synthesis: 73% was extracellular and 27% intracellular. However, warfarin no longer had its marked effect on intracellular prothrombin accumulation (Figs 7 and 8). Intracellular accumulation decreased from 82% to 42% (twofold, $P < .001$) whereas extracellular secretion increased from 19% to 59% (threefold, $P < .001$). This was confirmed by performing a Western blot analysis of extracellular and intracellular prothrombin without and with warfarin, obtained from samples incubated in the absence of ^{35}S -methionine (data not shown). Similar results were obtained with as well as without 10% FCS. This effect was

Table 1. Effect of Warfarin on Secreted Newly Synthesized Prothrombin

Experiment	2 h	Change (%)	4 h	Change (%)	24 h	Change (%)	P
1. Control (4)	0.672 ± 0.164	—	0.518 ± 0.126	—	—	—	—
2. Control (4)	0.274 ± 0.029	—	0.303 ± 0.053	—	—	—	—
Warfarin (4)	0.085 ± 0.030	-69	0.083 ± 0.009	-73	—	—	$<.004, <.007$
3. Control (4)	—	—	0.382 ± 0.043	—	0.376 ± 0.103	—	—
Warfarin (4)	—	—	0.161 ± 0.027	-58	0.301 ± 0.045	-20	$<.02, >.10$

Data are expressed as percentage of prothrombin synthesized $\pm$ SEM. Secreted newly synthesized total protein was calculated from radioactivity of excised radioactive bands on SDS-PAGE from 10 to 150 kd. Secreted newly synthesized prothrombin was calculated from radioactivity of the excised prothrombin band on SDS-PAGE of immunoprecipitated prothrombin, localized with added prothrombin marker. P value refers to differences between control and warfarin for 2 and 4 hours, respectively (experiment 2), or 4 and 24 hours, respectively (experiment 3), obtained by Student's *t* test, matched samples.

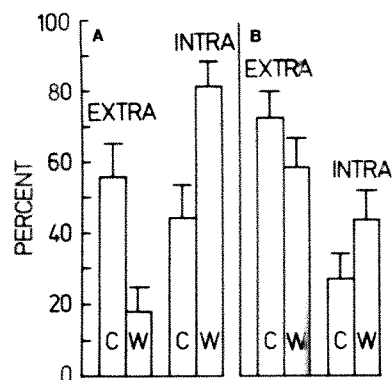


Fig 8. Quantitation of effect of warfarin on newly synthesized extracellular and intracellular prothrombin at 4 hours (panel A) and 24 hours (panel B). Percentage of extracellular and intracellular prothrombin was calculated from radioactive measurements obtained from excised extracellular and intracellular prothrombin bands after electrophoresis of prothrombin immunoprecipitates on 10% polyacrylamide SDS gels. C and W refer to control and warfarin incubations, respectively. SEM is given.

not due to inactivation of warfarin, since similar results were obtained with 5 $\mu\text{g}/\text{mL}$ warfarin; and warfarin in the media from the 24-hour warfarin incubation was still active when added to fresh Hep G2 cells for 4 hours (ie, accumulation of intracellular prothrombin and decrease in secretion of extracellular prothrombin, data not shown).

DISCUSSION

These data indicate that human hepatoma cells can secrete newly synthesized prothrombin of 83,000 daltons at a linear rate for 4 hours. Secreted prothrombin synthesis represented 0.3% to 0.7% of total secreted protein synthesis and was inhibitable by cycloheximide. Warfarin inhibited this secretion by 58% to 73%. Warfarin inhibition was accompanied by a parallel increase in the intracellular accumulation of a lower mol wt prothrombin species of 77,000 da.

The human hepatoma cell line Hep G2 has also been used by Fair and Bahnak,¹³ who analyzed its ability to synthesize and secrete several coagulation proteins, factor X, prothrombin, and antithrombin III. Secreted prothrombin had a mol wt of 73,000 by SDS-PAGE, 10,000 daltons lower than our findings. This discrepancy may be related to our use of the Laemmli procedure for SDS-PAGE,⁹ as compared with the Weber and Osborn¹⁴ technique used by Fair and Bahnak.¹³ Swanson and Suttie,¹⁵ using the Laemmli procedure, also recently reported a plasma mol wt of 83,000 for rat prothrombin. In addition, the same authors also noted a lower mol wt species of prothrombin in the microsomal fraction of 78,500 daltons, which accumulated with warfarin treatment. This lower intracellular mol wt species in the rat appears to be the same as the lower intracellular mol wt species that we noted in the human hepatoma cell line. Similar observations were reported in an abstract of Munns et al.¹⁶ Both intracellular species accumulate with warfarin and both are susceptible to endoglycosidase-H digestion, indicating the presence of early mannosyl residues that have

been incompletely processed. These precursors are found in the endoplasmic reticulum. Endoglycosidase-H will not digest the fully mature oligosaccharide complexes that have been processed by enzymes of the Golgi apparatus.¹⁷ This is substantiated by our observation that the secreted extracellular prothrombin species was not susceptible to endoglycosidase-H, as well as the observation of Swanson and Suttie that their higher mol wt species of 83,000 daltons was also not susceptible to endoglycosidase-H digestion. In addition, our data indicate that both the intracellular and extracellular species become identical in mol wt following deglycosylation with HF.

The effect of warfarin at 4 and 24 hours is of particular interest. After 4 hours of warfarin, low-mol-wt intracellular prothrombin accumulates markedly over control values in the presence of a marked decrease in extracellular secretion with no effect on total prothrombin synthesis. Thus, secretion has become rate-limiting. However, after 24 hours of warfarin, intracellular prothrombin decreased toward control levels in the presence of an insignificant decrease in extracellular secretion, with no effect on total prothrombin synthesis. Thus, an event has occurred following 24 hours of incubation, which increases the rate of intracellular prothrombin secretion despite the presence of warfarin. Under these conditions, extracellular prothrombin did not contain the lower mol wt prothrombin species. Thus, full and proper glycosylation of the intracellular prothrombin species was necessary for prothrombin secretion. This could not be explained on the basis of inactivation of warfarin or lack of viability of the hepatoma cell line, since this was not observed. Fair and Bahnak¹³ also observed no effect of warfarin on intracellular accumulation of prothrombin after 5 days of cell culture. These data are compatible with the hypothesis that glycosylation is linked to Vitamin K-dependent carboxylation of γ -glutamyl residues (ie, carboxylation is required for optimal carbohydrate processing), and that proper carbohydrate processing is required for optimal secretion of the prothrombin molecule. An apparent conflict with this interpretation is the observation of Swanson and Suttie¹⁵ that glycosylation was not essential for secretion of prothrombin from the liver following treatment of rats with tunicamycin (which prevents *N*-glycosylation of newly synthesized proteins). However, this does not rule out the possibility that a partially glycosylated molecule with incompletely processed carbohydrate chain may be incapable of being secreted from the liver. Furthermore, Swanson and Suttie¹⁵ did note a decrease in prothrombin secretion of tunicamycin-treated rats but explained this effect on the nonspecific decrease in total protein synthesis. Preliminary studies from our laboratory with tunicamycin treatment of Hep G2 cells indicate a 45% decrease in prothrombin secretion when data are expressed as percentage of total protein synthesis.

The reason for the changeover in secretion and intracellular accumulation between 4 and 24 hours remains to be established. Further experiments will be necessary to determine whether long-term warfarin treatment induces the formation of an enzyme(s) that enhances glycosylation or stimulates transport out of the endoplasmic reticulum. In

this regard, warfarin is capable of inducing at least one enzyme, the Vitamin K-dependent carboxylase of rat liver.¹⁸

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Long Lasting IgG Subclass and Antibacterial Polysaccharide Antibody Deficiency After Allogeneic Bone Marrow Transplantation

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Serum IgG subclasses were measured by a competitive indirect immunoassay with monoclonal antibodies in 31 leukemic patients before and after bone marrow transplantation. Antibodies to *Hemophilus influenzae* type b (Hib) capsular polysaccharide were determined in 28 cases. Abnormally low or borderline subclass (mostly IgG2 and IgG4) levels were found late after transplant in 23 infected and noninfected patients. These levels persisted for as long as 25 months, in association with low or borderline IgA levels in 78% of the cases. IgG2, IgG4, and IgA often showed a parallel evolution, whereas IgG1, IgG3, and IgM often varied together in the opposite way. Class but not subclass deficiencies were more frequent in patients with

graft-v-host disease (GVHD). Subclass abnormalities predominated in infected patients, with mean levels correlating with the severity of infections; however, the abnormalities are not clearly predictive of infections in individual cases. Most patients with Hib pneumonia showed virtually no IgG antibody response to Hib, and one-half of the patients had a moderate IgM and IgA response. In the whole series, many sera collected >1 year after graft contained very low or undetectable antibodies. Correlation between anti-Hib antibody and IgG2 levels was significant but weak because of discrepancies that were only partially explained by the subclass distribution of the antibodies.
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A COMBINED cellular and humoral immunodeficiency affects patients after allogeneic bone marrow transplantation (BMT). Reconstitution is slow, and certain long-term survivors suffer severe and/or recurrent infections. Serum immunoglobulin (Ig) classes may be depressed for ~1 year for IgG and IgM and even longer for IgA, with a correlation between infections, Ig class deficiency, and chronic graft-v-host disease (GVHD).¹⁻¹⁰ IgG subclass deficiency is a common feature of primary immunodeficiency states. It predominantly affects IgG2 and IgG4 and may be observed as a "selective" deficiency or in various immunodeficiency syndromes,¹¹ especially IgA deficiency occurring as an apparently isolated defect¹²⁻¹⁷ or in ataxia telangiectasia.¹⁸⁻²¹ A frequent pathogen in such patients is *Hemophilus influenzae* type b (Hib),^{11,22,23} whose major antigen is the capsular polysaccharide polyribosylribitolphosphate (PRP).²⁴ This is not surprising, since IgG2 appears to be the predominant subclass of antibacterial carbohydrate antibodies (Ab).²⁵⁻²⁷ The observation of a relatively high incidence of Hib pneumonia in our transplanted patients²⁸ led us to study serum IgG subclass and anti-PRP Ab levels in transplanted patients affected with Hib pneumonia. Sera from patients with other infections or without infections were studied as controls.

PATIENTS AND METHODS

Patients. Nine patients who received allogeneic BMT and experienced Hib pneumonia were retrospectively selected for the study. In view of the results, 22 other transplanted patients who survived >90 days and in whom serial posttransplant sera were available also were chosen as control cases. None of the patients received any IgG therapy during the study. None of the control patients had experienced Hib pneumonia, and we purposely selected patients with or without GVHD, viral, or other bacterial infections. Consequently, patients were listed according to the nature of their infections (Table 1): patients infected with extracellular bacteria, mostly Hib (compatible with impaired humoral immunity) (7 cases); patients with viral and/or intracellular bacterial infections (suggestive of cellular immune defect) (10 cases; two further patients suffered pneumonia of unknown origin); patients with both types of infections (5 cases), including Hib pneumonia in 2 cases; and patients free of infections (7 cases). Chemotherapy regimens preceding the graft varied according to the type and staging of the leukemias and to the number

of relapses. The conditioning regimen uniformly consisted of cyclophosphamide (60 mg/kg on days -5 and -4) and total body irradiation (10 gray, with lung shielding above 8 gray). To prevent GVHD, methotrexate was given until day 102 to all patients receiving allogeneic BMT according to the Seattle regimen.¹ Furthermore, patient 16 received T-depleted bone marrow. Patient 29, the only patient who had received a syngeneic transplant, was not submitted to GVHD prophylaxis. GVHD was diagnosed and graded according to usual criteria.^{29,30} No noninfected patient suffered GVHD. In contrast, most patients with repeated infections were affected with severe chronic GVHD.

Measurement of immunoglobulin class and subclass levels. Ninety-six sera were collected before transplant and after BMT (follow-up 4 to 25 months, mean 13.2 months). Ig class levels were measured by laser nephelometry. IgG subclass levels were determined in coded samples by a competitive indirect immunoassay (ELISA) with monoclonal Ab, as previously described.^{31,32} Normal values^{15,31,32} were established in a study of 129 to 186 sera from normal blood donors aged 20 to 50 years. Serum of the donors of bone marrow grafts was not examined. Values below or at the lower limit of the 95% percentile range of normal sera (ie, 4.0 mg/mL, 0.6 mg/mL, and 0.18 mg/mL for IgG1, IgG2, and IgG3; 6.1, 0.8, and 0.5 mg/mL for IgG, IgA, and IgM, respectively) are described below as subclass or class deficiencies. For IgG4, whose level differs according to sex,³¹ a 95% percentile range limit can be defined in men only (0.03 mg/mL). IgG2 and IgG4 display very heterogeneous distributions in healthy subjects, and low normal values are likely to

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include deficiencies.³² Borderline levels (≤ 0.95 mg/mL for IgG2 and < 0.1 mg/mL in both sexes for IgG4) are therefore considered in the analysis.

Determination of anti-PRP antibody levels. Anti-PRP Ab were measured in 79 sera from 28 patients (mean time between BMT and collection of the last serum studied was 14.3 months). PRP was prepared from culture supernatants by precipitation with a cationic detergent and further purification by saline and phenol extractions³³ and was coupled to tyramine according to the method of Anthony et al.³⁴ Microtitration plates were coated with PRP-tyramine (5 μ g/mL PRP in phosphate-buffered saline [PBS]) and saturated with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20. One-half of every plate was treated identically except that PRP was replaced by PBS (blanks). Sera diluted $1/20$ in 0.5% BSA containing PBS-Tween were incubated for 90 minutes at 37°C in triplicates in the PRP-coated and control wells. The plates were washed six times in PBS-Tween and incubated with peroxidase-tagged goat antisera specific for human γ , α , and μ Ig chains (Institut Pasteur Production, Paris). After washings, bound conjugate was revealed with α -phenylenediamine and H2O2, and optical densities (OD) were recorded on a microplate reader.³¹ Each ELISA plate contained standard positive and negative sera, and, as a reproducibility test, three further known positive sera (interassay coefficients of variation were 9.8% for IgG Ab, 7.8% for IgA Ab, and 11.3% for IgM Ab). For every sample, the experiment was repeated on the same plate with serum previously incubated for 45 minutes with high-concentration (500 μ g/mL) PRP. Only specific OD (inhibited by PRP) were taken into account. Results were expressed in arbitrary units calculated from specific OD in PRP-coated wells after results on uncoated wells were deducted by comparing the same data with the positive and negative standard sera.

Due to lack of material, the subclass distribution of IgG anti-PRP Ab could be studied in four patients only. These experiments were performed by a procedure similar to that previously described except that bound anti-PRP Ab were revealed by subclass-specific mono-

clonal Ab followed by peroxidase-coupled rabbit IgG anti-mouse IgG extensively absorbed with human IgG, as described in detail previously.³⁵

Statistical analysis. Ig and anti-PRP Ab levels in patients and controls were compared by the Student's *t* test when possible, or by the Mann and Whitney's test when the number of subjects was too small. Percentages were compared using the Chi-square test.

RESULTS

Detailed results of measurement of Ig class and subclass and anti-PRP antibody levels are shown in Table 1. Before transplant, several patients had low (patients 4, 7, 8, and 26) or borderline (patients 18 and 19) IgG2 and/or IgG4 (patients 1, 2, 3, 6, 24, 28, and 31) levels. IgA and IgG3 were rarely depressed (patients 1, 5, and 28, respectively), whereas abnormally low IgM levels were noticed in patients 1, 4, 8, 12, and 18. None of these patients had any past history of abnormal infections. The evolution of the levels of the various Ig isotypes after BMT varied greatly from patient to patient. However, comparison of the curves drawn for each patient (not shown), revealed a strong tendency for certain isotypes to vary together: IgG1 and IgG3 showed a roughly parallel evolution in 68% of cases, IgG1 and IgM in 48% of cases, and IgG3 and IgM in 45% of cases. On the other hand, roughly parallel curves were observed for IgG2 and IgG4 (61% of cases), IgG2 and IgA (52% of cases), and IgG4 and IgA (48% of cases). Any other association between Ig isotypes was noted in $< 19\%$ of cases. In contrast, IgG1 and IgG2 varied in the opposite way in 61% of cases. Consequently, IgG2 levels were lower in late (> 350 days after transplant) than in early samples in 11 cases. Subclass deficiencies or borderline levels at the end of follow-up

Table 1. Diagnosis, Infection Patterns, Serum Ig, and Anti-PRP Ab Levels

Patient No.	Age/Sex (yr)	Donor (Age/Sex)	Diagnosis	GVH		Infections	Date	Serum Ig (mg/mL)							Anti-PRP Ab (U)		
				Acute	Chronic			IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4	IgG	IgA	IgM
1	28/M	25/M	AML, 1st CR	0	0	<i>H influenza</i> (pneumonia) (d 120)	d -7	7.3	0.15	0.24	7.1	1.25	0.28	0.045	33	5	3
							d 169	15.8	0.85	0.88	13.3	0.75	0.37	0.01	—	—	—
							d 401	21.6	0.84	2.78	20.2	0.05	0.34	0.09	14	4	14
2	20/F	24/M	AML, 1st CR	Grade II	+	<i>H influenza</i> (pneumonia) (d 352) <i>U</i> (pneumonia) (d 450)	d -8	11.4	0.95	0.86	11.5	2.3	0.7	0.05	37	18	22
							d 7	10.9	1.24	0.91	7.9	1.6	0.56	0.07	—	—	—
							d 504	11.6	0.53	1.24	9.1	1.4	0.6	0.03	14	6	9
3	33/F	39/M	CML, chronic phase	0	++	<i>H influenza</i> (pneumonia) (d 248)	d -3	10.6	2.74	1.83	6.6	3.1	0.41	0.002	70	63	32
							d 253	M	0.7	4.15	42.5	1.55	1.05	0.08	8	5	12
							d 392	M	0.9	2.11	31	1.6	0.25	0.039	2	18	1
4	4/M	6/M	AML, 2nd CR	0	0	<i>H influenza</i> (pneumonia) (d 240)	d -9	6.5	1.01	0.3	5.8	0.2	0.32	0.22	4	—	—
							d 267	6.4	0.65	0.41	8.5	1.15	0.49	0.124	17	26	22
							d 518	5.3	0.49	0.81	7.5	0.85	0.21	0.108	33	20	30
5	31/F	34/M	AML, 2nd CR	Grade II	0	<i>H influenza</i> (pneumonia) (d 380) <i>U</i> (pneumonia) (d 580)	d -6	11.3	2.0	1.08	9.0	1.4	0.04	0.21	23	44	11
							d 750	29.0	1.29	2.28	34.0	1.8	0.45	0.20	—	—	—
							d 750	29.0	1.29	2.28	34.0	1.8	0.45	0.20	—	—	—
6	16/M	14/F	AML, 1st CR	Grade I	+	<i>K pneumonia</i> (pneumonia) (d 167)	d -8	8.0	1.83	0.47	6.2	2.7	0.54	0.09	47	3	8
							d 68	3.8	0.2	0.29	3.8	0.9	0.33	0.03	19	2	2
							d 166	4.3	0.2	M	5.4	0.85	0.15	0.05	0	3	4
7	25/M	32/F	AML, 1st CR	Grade III	+	<i>H influenza</i> + <i>K pneumonia</i> (pneumonia) (d 77) <i>H influenza</i> + <i>Asp</i> + <i>S aureus</i> (pneumonia) (d 180)	d -8	11.5	1.82	1.1	—	—	—	—	—	—	—
							d 0	—	—	—	4.3	0.3	0.31	0.11	1	5	14
							d 140	M	0.27	0.37	6.0	0.75	0.37	0.07	10	0	2
8	9/M	5/M	ALL, 1st RC	0	0	CMV (pneumonia) (d 70) <i>Herpes-zoster</i> (d 330)	d 176	M	0.19	0.28	2.9	0.75	0.3	0.05	10	0	18
							d 0	5.0	0.5	0.12	5.2	0.5	0.3	0.41	9	12	4
							d 252	9.2	0.35	0.73	10.9	0.3	0.43	0.05	4	7	3
9	10/M	12/F	ALL, 2nd CR	0	0	CMV (pneumonia) (d 107)	d 8	8.5	1.5	0.55	7.3	2.0	0.22	0.03	11	11	4
							d 169	7.7	0.7	0.46	8.6	0.8	0.26	0.08	10	5	2
							d 363	9.6	0.93	0.93	8.75	1.4	0.4	0.59	3	7	5
10	21/M	20/F	AML, 1st CR	0	++	<i>L. monocytogenes</i> (septicemia) (d 204) <i>CMV</i> (pneumonia) (d 410) <i>L. pneumonia</i> (pneumonia) (d 690) <i>PV</i> (encephalitis) (d 755)	d -7	12.8	3.72	0.77	9.75	4.3	0.69	1.8	19	—	—
							d 405	M	0.36	M	7.5	0.12	0.57	1.4	5	—	—
							d 760	M	0.52	M	6.6	0.55	0.20	0.24	7	1	9

Table 1. Diagnosis, Infection Patterns, Serum Ig, and Anti-PRP Ab Levels (Cont'd)

Patient No.	Age/Sex (yr)	Donor (Age/Sex)	Diagnosis	eVH		Infections	Date	Serum Ig (mg/mL)								Anti-PRP Ab (U)		
				Acute	Chronic			IgG	IgA	IgM	IgG1	IgG2	IgG3	IgG4	IgG	IgA	IgM	
11	26/M	30/M	ALL, 1st CR	0	+	CMV (pneumonia) (d 90)	d -7	14.0	1.55	0.9	11.5	5.0	0.7	2.4	---	---	---	
							d 12	11.0	1.61	1.1	7.6	4.25	0.38	1.53	26	7	16	
							d 128	M	0.69	0.35	9.2	1.4	0.21	0.25	4	1	18	
							d 273	M	0.68	1.07	9.1	2.4	0.50	0.33	6	1	10	
12	22/M	27/M	AML, 1st CR	Grade I	+	Varicella (d 30) CMV (pneumonia) (d 131)	d -7	9.8	0.81	0.32	---	---	---	---	---	---		
							d 0	9.8	0.67	0.25	6.6	4.3	0.23	0.24	13	4	4	
							d 112	8.5	0.3	1.4	5.7	2.2	0.43	0.06	16	8	10	
							d 240	M	0.27	M	14.6	0.9	1.10	0.04	13	3	3	
13	42/M	35/F	AML, 1st CR	Grade II	0	CMV (pneumonia) (d 120) Herpes-zoster (d 240)	d 7	9.0	1.24	0.87	6.4	2.2	0.25	0.18	35	10	9	
							d 154	9.3	0.57	0.89	9.9	0.8	0.85	0.06	25	9	5	
							d 700	9.2	0.68	0.69	8.25	0.9	0.57	0.34	23	4	9	
14	33/F	35/M	AML, 1st CR	0	0	CMV (pneumonia) (d 145)	d -8	9.7	1.67	2.72	9.35	2.7	0.55	0.26	---	---	---	
							d 153	10.7	1.17	1.42	17.5	1.55	0.61	0.13	---	---	---	
							d 291	10.0	0.74	2.7	15.1	2.75	0.57	0.13	---	---	---	
15	35/M	39/F	AML, 1st CR	0	0	Herpes-zoster (d 90)	d -9	11.6	0.88	1.04	10.7	3.15	0.84	0.43	101	26	34	
							d 81	9.2	0.48	0.34	11.5	1.6	0.72	0.16	12	57	4	
							d 216	11.7	0.6	1.6	13.4	0.3	---	0.014	6	9	6	
16	41/F	38/F	ALL, 1st CR	0	0	Varicella (d 145)	d 8	3.8	0.51	0.33	3.15	0.65	0.32	0.119	2	0	4	
							d 68	8.3	0.64	0.23	6.75	1.45	0.43	0.215	7	0	4	
							d 230	M	0.68	0.49	9.1	0.95	0.35	0.094	4	0	3	
17	15/M	12/M	ALL, 1st CR	0	0	Herpes-zoster (d 185) Uti (pneumonia) (d 355)	d 5	7.4	0.87	0.41	5.7	1.85	0.27	0.16	13	---	---	
							d 377	10.5	0.68	1.13	11.1	1.0	0.76	0.23	4	11	11	
							d 474	7.6	0.41	0.72	7.0	1.2	0.58	0.17	14	---	---	
18	34/M	28/F	ALL, 4th CR	Grade II	+	Uti (pneumonia) (d 39) Uti (pneumonia) (d 164)	d -8	8.2	0.7	0.2	7.3	0.75	0.33	0.33	---	---	---	
							d 82	4.9	0.25	0.29	3.5	0.6	0.24	0.15	---	---	---	
							d 353	M	0.22	0.29	15.7	0.25	0.24	0.026	---	---	---	
19	22/F	20/F	AML, 1st CR	Grade II	+	Uti (pneumonia) (d 120)	d -5	17.9	1.14	1.58	15.7	0.92	1.37	0.39	131	64	32	
							d 358	8.6	0.26	0.92	9.1	0.35	1.25	0.12	175	11	5	
							d 102	2.4	0.13	M	2.65	0.6	0.28	0.04	25	13	14	
20	28/M	29/F	CML, chronic phase	0	++	Herpes-zoster (d 310) AV (pneumonia) (d 360) Str. pn (meningitis) (d 520) Herpes-zoster (d 790) E. coli (septicemia) (d 855) Mumps (orchitis) (d 920)	d 385	2.9	0.3	0.27	2.65	0.35	0.34	0.05	---	---	0	
							d 432	3.6	0.25	M	4.0	0.05	0.50	0.05	0	0	0	
21	27/F	24/F	AML, 1st CR	Grade IV	+	CMV + Str. pn (pneumonia) (d 133)	d -8	15.6	0.8	1.3	15.8	4.1	0.43	0.40	---	---	---	
							d 109	M	0.29	M	4.4	0.85	0.36	0.04	---	---	---	
							d 119	---	---	---	5.25	0.60	0.25	0.03	---	---	---	
							d -8	14.3	1.8	2.67	16.5	1.5	0.75	0.48	61	40	77	
22	35/M	32/M	AML, 1st CR	Grade II	+	Herpes-zoster (d 185) H. influ (pneumonia) (d 369)	d 81	4.1	0.19	0.2	3.6	0.75	0.21	0.18	14	0	17	
							d 384	11.2	0.45	1.86	12.0	0.01	0.58	0.01	5	4	11	
							d 662	13.4	0.41	1.82	12.0	0.05	0.55	0.045	4	1	27	
							d 74	---	---	---	6.5	1.0	0.3	0.28	---	---	---	
23	29/F	30/M	CML, chronic phase	0	0	Herpes-zoster (d 125) H. influ (pneumonia) (d 180) chronic sinusitis (d 195)	d 204	M	0.61	1.68	10.8	0.8	0.52	0.026	1	9	19	
							d 285	18.8	1.19	1.35	21.3	0.55	0.64	0.09	3	---	27	
							d -7	9.9	1.61	1.53	8.5	1.45	2.7	0.016	---	---	6	
24	20/F	20/M	AML, 1st CR	0	0	varicella (d 370) H. influ (pneumonia) (d 760)	d 377	M	0.95	1.43	28.9	0.75	0.68	0.058	34	11	3	
							d 770	12.5	1.28	1.0	19.4	0.4	0.9	0.065	34	114	1	
							d -8	8.9	1.63	0.47	8.0	2.5	0.84	0.20	32	37	11	
25	11/M	14/F	AML, 1st CR	0	0	---	d 77	6.1	0.98	0.32	1.7	1.7	0.60	0.063	14	14	7	
							d 372	9.3	0.98	1.56	4.4	1.46	1.35	0.09	5	4	8	
							d -8	17.6	1.19	0.74	12.7	0.6	0.90	0.06	56	1	27	
26	15/F	17/M	AML, 1st CR	0	0	---	d 61	7.8	0.39	0.51	9.4	2.3	0.01	0.19	42	---	---	
							d 369	6.6	0.51	0.42	6.1	0.85	0.65	0.04	3	0	1	
							d -7	8.3	1.14	1.03	6.4	2.0	0.47	0.17	35	19	15	
27	16/F	14/M	ALL, 1st CR	0	0	---	d 97	5.0	0.2	0.43	4.3	0.7	0.24	0.07	16	0	1	
							d 363	M	0.30	0.71	12.4	0.95	0.33	0.009	0	0	2	
							d -8	9.2	0.71	0.54	7.2	2.4	0.01	0.029	4	4	12	
28	30/F	14/M	AML, 1st CR	0	0	---	d 222	12.8	1.35	7.9	11.1	0.4	0.9	0.032	0	4	11	
							d 450	7.2	2.07	1.52	7.2	1.7	1.3	0.13	3	5	7	
							d -118	15.3	2.15	2.33	16.5	2.9	2.1	1.11	31	70	51	
29	25/M	Syngeneic graft	CML, acute phase	0	0	---	d 134	13.8	1.3	1.61	10.1	3.1	1.35	0.27	69	61	8	
							d 293	9.9	0.96	0.69	11.1	2.9	1.1	0.185	35	51	14	
							d -8	7.0	1.51	0.96	6.2	1.1	0.39	0.11	56	47	23	
30	27/M	25/M	ALL, 1st CR	0	0	---	d 91	7.2	1.24	0.2	7.6	2.6	0.51	0.09	34	18	15	
							d 217	8.0	1.37	1.2	8.7	2.2	0.49	0.048	45	21	18	
							d -8	9.0	0.7	0.54	6.5	1.5	0.38	0.065	13	3	7	
31	24/F	39/F	ALL, 1st CR	0	0	---	d 8	7.8	0.92	0.53	5.0	1.75	0.35	0.08	---	---	---	
							d 113	7.9	0.1	0.23	3.5	1.0	0.39	0.037	13	0	4	
							d 275	8.1	0.37	0.65	8.1	0.9	0.65	0.077	5	0	---	
							d 522	M	---	---	6.7	0.7	0.62	0.08	0	0	---	

predominantly affected IgG2 (20 patients) and IgG4 (19 patients), together with low or borderline IgA levels in 78% of cases.

The analysis of subclass levels in the last studied samples showed that long-term subclass deficiencies were observed predominantly in infected patients (only one noninfected patient had IgG4 deficiency). Every patient with an infection pattern compatible with both cellular and humoral ID was subclass-deficient, with a maximum follow-up of 25 months. Borderline levels were observed in all patients' groups, including noninfected subjects, in whom the incidence of complete recovery was the highest. Differences according to the infection patterns are reflected in the mean IgG subclass values in the last studied samples (Table 2). On the whole, IgG1 and IgG3 were higher and IgG2 and IgG4 lower than in normal subjects (differences between patients and controls were significant except for IgG3 levels in infected patients); the lowest values for the two latter isotypes were in patients with infections of both types (mean 0.33 and 0.08 mg/mL, respectively) and the highest in noninfected patients. Because of the small size of the group of noninfected patients, differences between infected and noninfected patients were significant for IgG2 levels only ($P < .05$). No significant correlation was observed between the ages of the donors and recipients of the graft and the incidence of subclass deficiency.

Serum collected at periods close to infectious episodes was available in certain patients only. Comparison with subclass levels in the sera from noninfected patients showed an incidence of subclass deficiency possibly higher in infected patients (the difference is not significant in such a small series). Indeed, for IgG2, low levels were observed during 8 of 21 infectious episodes (4 with extracellular bacteria and 4 with intracellular bacteria or viruses) and borderline levels during 6 other episodes, whereas of 14 samples collected from noninfected subjects at similar periods after graft, one contained low and 5 contained borderline IgG2 levels. Despite this, the finding of normal subclass levels during one-third of the infectious episodes and the rare but possible occurrence of low levels in noninfected patients make the finding of subclass deficiency suggestive but not predictive of occurrence of infection in individual cases.

Comparison of serum Ig levels in patients with and without GVHD clearly shows that class deficiencies occurring late after BMT were observed predominantly in chronic GVHD: Only patients with chronic GVHD had total IgG and IgM deficiencies, and IgA deficiency was more frequent

in GVHD (75% v 32% of cases, $P < .02$). As for IgG subclasses, the incidence of deficiency did not differ according to the presence or absence of acute or chronic GVHD. However, the patient who received a syngeneic graft (patient 29) is one of the only two patients in whom every isotype was at normal or high levels throughout the study.

Six of nine patients affected with Hib pneumonia had abnormally low or borderline serum IgG2 levels, an incidence that is not different from that observed in patients in whom Hib pneumonia could not be demonstrated. This led us to determine serum levels of anti-PRP Ab. Before transplant, patients 4 and 7, who had low IgG2, also had low Ab levels (Table 1). Mean Ab levels before graft, calculated excluding patients with IgG2 deficiency, were similar in the different patient groups and comparable to those in normal adults. We had enough material to study anti-PRP Ab at the moment of Hib pneumonia in only two patients (patients 3 and 7); their levels were low although IgG2 was in the normal range in case 3. After Hib infection, IgG anti-PRP Ab remained low or continued to decrease except in patients 4 and 24, in whom they reached levels comparable to those in normal subjects without known Hib infection. Patient 7, who showed a poor response after Hib pneumonia, experienced a second bout of Hib infection 3 months later. Mean IgG anti-PRP Ab level was not higher in patients infected with Hib than in the other patients under study (Table 3). Patients 4 and 24 had low IgG2 levels. Due to lack of material, we could not study the subclass distribution of these IgG Ab. This experiment was performed in patient 1 at day 401; the patient's serum contained subthreshold levels of IgG2 and low but detectable IgG anti-PRP Ab. Two-thirds of these Ab belonged to the IgG1 subclass, and one-third belonged to IgG2.

A moderate increase in IgM and IgA Ab was observed just after the pneumonia in four and three cases, respectively. A strong IgA response was manifested by one case. Consequently, mean IgM and IgA Ab levels after Hib infection were similar to normal subject levels (Table 3).

Most patients without known Hib infection had low anti-PRP Ab of the three classes, the latest samples studied often showing the lowest values. A number of sera collected after 1 year contained no detectable Ab or levels similar to those in young children (Tables 1 and 3). However, there were exceptions, such as patients 19, 29, and 30. Patient 19 had high Ab levels before transplant and 1 year later, despite a very low IgG2 level. This striking contrast led us to study the subclass of the Ab in the sample collected at day 358. In fact, we found that anti-PRP Ab in this serum was restricted to

Table 2. Serum IgG Subclass Levels in BMT Patients and Controls

Subjects	n	IgG1	IgG2	IgG3	IgG4
BMT					
All patients					
Before graft	25	9.24 $\pm$ 3.63	2.12 $\pm$ 1.33	0.66 $\pm$ 0.59	0.39 $\pm$ 0.56
Last sample	31	11.49 $\pm$ 7.19	1.01 $\pm$ 0.77	0.58 $\pm$ 0.33	0.12 $\pm$ 0.12
Infected patients, last sample	24	12.49 $\pm$ 7.94	0.86 $\pm$ 0.73	0.50 $\pm$ 0.28	0.125 $\pm$ 0.135
Noninfected patients, last sample	7	8.09 $\pm$ 2.63	1.54 $\pm$ 0.74	0.83 $\pm$ 0.38	0.08 $\pm$ 0.06
Normal adults	129-186	6.43 $\pm$ 1.50	2.64 $\pm$ 1.36	0.42 $\pm$ 0.17	0.39 $\pm$ 0.38

Values are mean $\pm$ SD, mg/mL.

Table 3. Anti-PRP Ab Levels in the Last Studied Samples From BMT Patients and in Control Sera

Subject	IgG			IgA			IgM		
	n		Range	n		Range	n		Range
BMT									
All patients	27	17	(0-175)	25	12	(0-114)	25	9	(0-30)
<i>Hemophilus influenzae</i>	8	14	(2-34)	7	23	(0-114)	8	16	(1-30)
No <i>Hemophilus influenzae</i>	19	18	(0-175)	19	7	(0-51)	19	6	(0-18)
Normal children aged 12-25 mo	10	3	(0-10)	10	2	(0-12)	10	8	(2-15)
NHS pool*	1		60	1		21	1		21
Normal adults									
Before	5	32	(0-76)	5	33	(10-71)	3	33	(29-35)
3 weeks after vaccination†	5	113	(36-235)	5	169	(39-323)	5	73	(44-100)

Values are units, means, and ranges. Ab levels in patients were significantly lower than in normal (nonvaccinated) subjects. Due to the small size of the groups, differences between Hib-infected and non-Hib-infected patients are not significant.

*Normal human sera from 60 adult blood donors.

†One injection of PRP (25 µg).

IgG2. Two other sera with fair Ab and low IgG2 levels were studied for subclass distribution (patient 20 at day 102 and patient 26 at day -8), and the Ab were equally distributed in the IgG1 and IgG2 isotypes. The correlation between IgG2 and anti-PRP Ab in the whole study, although significant ($P < .05$), was weak ($r = .23$).

DISCUSSION

The present study shows that IgG subclass deficiencies occur with a very high frequency in long-term survivors after BMT. They predominantly affect IgG2 and IgG4, as in a variety of primary and acquired immunodeficiency syndromes.^{12,15} Similar findings were recently reported in abstract form.³⁶ A striking observation in our study is that IgG2-IgG4 deficiencies, often associated with low IgA levels, frequently developed late after transplant, whereas IgM, IgG1, and IgG3 and total IgG had reached normal or high levels. Indeed, IgG2, IgG4, and IgA often showed a parallel evolution, whereas IgG1, IgG3, and IgM tended to vary together in the opposite way. This is reminiscent of several observations: in childhood, the increase of serum IgG1 and IgG3 levels is rapid and follows total IgG, whereas IgG2 and IgG4 reach adult levels only around puberty (as IgA does)^{37,39}; in common variable immunodeficiency subclass imbalance is very common, with predominant deficiencies of either IgG2 and IgG4 or IgG1 and IgG3¹⁵; patients with "selective" IgA deficiency often have IgG2-IgG4 deficiency, with frequent IgG1-IgG3 increase in infected patients.¹²⁻¹⁷ These subclasses also differ with respect to Ab specificity, with an IgG1-IgG3 predominance of Ab to viral proteins⁴⁰⁻⁴³ and an IgG2 predominance of antibacterial polysaccharide Ab.²⁵⁻²⁷ Finally, studies of in vitro B cell maturation are compatible with different regulations of IgG2-IgG4 and IgG1-IgG3 expression.⁴⁴⁻⁴⁶

The asynchronous evolution of the subclasses and the well-known existence of profound T cell disturbances after BMT^{1-6,47-50} suggest that subclass deficiency might result from an impaired T cell regulation rather than from an intrinsic B cell defect. Experimental work in the mouse^{51,52} favors the hypothesis that the expression of the isotypes

whose genes are located downstream in the C_H locus (as IgG2 and IgG4 are in humans) requires more T cell help than does control of upstream isotypes. The finding of IgG2-IgG4 deficiency in human syndromes featuring impaired T-B cell cooperation, such as the Di George's syndrome, the severe combined immunodeficiency syndrome with abnormal expression of HLA class II antigens, ataxia telangiectasia, and AIDS^{15,19,21,53} is in keeping with this hypothesis. Although bacterial polysaccharides are T-independent antigens, antipolysaccharide Ab production is highly dependent on T cell regulation.⁵⁴ Therefore, both subclass and anti-PRP Ab deficiencies may result from abnormal T cell function. Cells from five of the present patients were studied for in vitro suppressor activity.⁵⁰ There is no correlation between excessive suppressor activity and the present findings, suggesting that impaired helper function might play a major role.

Previous work¹⁻¹⁰ showed that GVHD is a significant factor in the occurrence of infections and of Ig class deficiency; this is confirmed in the present study. In contrast, we observed an incidence of subclass deficiency similar in patients with and without GVHD. However, the finding of fully normal Ig levels throughout the study was exceptional and concerned virtually only the patient who received a syngeneic transplant. Therefore, that clinically undetectable GVHD plays a role in the pathogenesis of subclass deficiencies is a possibility. Both the mean subclass levels and incidence of subclass deficiencies differed according to the infection patterns. Subclass deficiencies were observed in patients infected with extracellular bacteria as well as in patients with viral or intracellular bacterial infections in whom they are unlikely to play a direct role. They were constant in patients with both types of infections and may reflect the general level of immunodeficiency in these patients. The subclass deficiencies may contribute to the occurrence of extracellular bacterial infections, but certain patients free of infections had low or borderline subclass levels, whereas some infected patients had normal class and subclass levels at the period of infectious episodes. The explanation of the occurrence of infections is therefore more

complex than a mere subclass deficiency, even in patients with Hib pneumonia. Anti-PRP Ab defect might play some role, since we observed low levels just before pneumonia in a patient with normal IgG2 level and since there was a very poor IgG Ab response after pneumonia (protection against Hib is predominantly mediated by IgG Ab⁵⁵). One patient with low IgG response experienced a second bout of Hib pneumonia 3 months later. Several patients who had no Hib pneumonia developed anti-PRP Ab (patients 19, 24, 29, and 30), but we do not know whether the other patients without pneumonia have been infected by Hib. Humoral immunodeficiency thus probably plays a role in the genesis of infections observed in BMT recipients. Thus, substitutive IgG therapy of infectious episodes may be indicated even in patients without class and/or subclass deficiency. Whether a prolonged systematic IgG treatment is indicated for prophylaxis of infections remains to be determined. On the other hand, the high incidence of Hib pneumonia in our series²⁸ and the defective anti-PRP Ab response raises the questions of the indication and timing of vaccination in transplanted patients. In such cases, a protein conjugate PRP vaccine should be used, since it is effective in IgG2-deficient patients who do not respond to PRP alone.⁵⁶

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1986 Update of HIV Seroprevalence, Seroconversion, AIDS Incidence, and Immunologic Correlates of HIV Infection in Patients With Hemophilia A and B

Margaret V. Ragni, Alan Winkelstein, Larry Kingsley, Joel A. Spero, and Jessica H. Lewis

A cohort of 181 patients with hemophilia A (149) and hemophilia B (32) cared for at the Hemophilia Center of Western Pennsylvania was followed to determine human immunodeficiency virus (HIV) seroprevalence, seroconversion rate, and clinical and immunologic correlates of HIV infection. By December 1986, 82 (45%) were HIV seropositive, and of these, ten (12%) had developed AIDS, 28 (34%) had symptomatic HIV infection (CDC class III, IV), of whom 14 (17%) had AIDS-related complex (ARC), and 44 (54%) had asymptomatic HIV infection (CDC class II). The HIV seropositive group included 82% of those treated with factor VIII concentrate (97% severe, 5% moderate), 48% of those treated with factor IX concentrate (92% severe, 8% moderate), 10% of those treated with cryoprecipitate (67% severe, 33% moderate), and none of those treated with

fresh frozen plasma. Based on 77 serially sampled HIV seropositive hemophiliacs (1977 to 1986), peak seroconversion occurred in 1982, with 14% (11 of 77) occurring since 1984. With increasing time from seroconversion, both T_4 lymphocyte number and function (the latter measured by growth in soft agar [T colony assay]) progressively declined; T_4 number declined to $135 \pm 26/\text{mm}^3$ (SEM), and colony count declined 1193 ± 537 (control 3851 ± 387) by 5 years after seroconversion. In those developing AIDS, total T_4 fell below $100/\text{mm}^3$ ($33 \pm 8/\text{mm}^3$) at diagnosis. In this cohort, the overall AIDS incidence is 5.5% (12% among the HIV seropositive) and in those seropositive 5 or more years, the AIDS incidence approaches 32%.

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ALTHOUGH FEWER than 1% of the total number of AIDS cases occur in hemophiliacs, the incidence of AIDS in this group is quite high (greater than one case per 100).^{1,2} Moreover, AIDS has become a leading cause of death in this group.³

Hemophiliacs differ from other high risk groups not only in the rarity of sexually transmissible diseases and intravenous (IV) drug use but also in the expected cessation of further human immunodeficiency virus (HIV) exposure because of current HIV antibody donor screening and heat inactivation of blood products. Although a recent Centers for Disease Control (CDC) survey² has shown a decline in the rate of AIDS cases in hemophiliacs since mid-1985, the long-term prognosis of HIV-infected hemophiliacs is not yet known, since the peak in seroconversion in hemophiliacs occurred in 1982, and the interval from infection to development of AIDS may be 5 years or longer.^{4,5}

Previous study in this laboratory has revealed that hemophiliacs with AIDS-related illness show impaired cellular immunity, specifically, depressed lymphocyte function as measured by in vitro T lymphocyte growth in soft agar (T colony assay). Although immune parameters that predict the development of AIDS are unknown, a recent study has shown that a significant decrease in T_4 helper lymphocytes in HIV antibody-positive homosexuals, drug users, and hemo-

philiacs is associated with an increased risk for the development of AIDS.⁶

In this study, we report the seroprevalence, seroconversion, AIDS incidence, and immunologic correlates of HIV infection in a cohort of 182 hemophiliacs from western Pennsylvania.

MATERIALS AND METHODS

Clinical data. The cohort studied here has been previously reported in detail elsewhere.⁴ One hundred fifty had classic hemophilia or hemophilia A (congenital factor VIII:C deficiency), 79 of whom were treated with factor VIII concentrate, 57 with cryoprecipitate, and 14 (who had inhibitors to factor VIII) with factor IX concentrate. The remaining 31 had Christmas disease or hemophilia B (congenital factor IX:C deficiency), 14 of whom were treated with factor IX concentrate and 17 with fresh frozen plasma. By late 1985, 13 treated with cryoprecipitate and three treated with fresh frozen plasma were switched to heat-treated factor VIII or factor IX concentrate, respectively. There were no seroconversions in any of these patients, and for purposes of this study they are classified by their previous blood product exposure, ie, cryoprecipitate or fresh frozen plasma. Those patients classified as cryoprecipitate-treated or fresh frozen plasma-treated received only those products; they were never treated with lyophilized concentrates. Once a patient began lyophilized concentrate, he was classified as a concentrate-treated patient. The patients were classified according to the Public Health Service classification system for HIV infection⁷: acute infection, class I; asymptomatic infection, class II; lymphadenopathy, class III; and AIDS, AIDS-related complex (ARC), neurologic symptomatology, herpes zoster, or oral candidiasis, class IV. Lymphadenopathy is defined as lymph node enlargement (≥ 1 cm) in greater than two extrainguinal areas for greater than 3 months.

Plasma samples. Citrated plasma samples were obtained from all 181 patients during routine clinical evaluation. In addition, 77 of the 82 HIV seropositive hemophiliacs on whom samples were available annually (66 [86%]) or biannually (11 [14%]) between 1977 and 1986 constituted the population base for determining seroconversion rate and AIDS incidence. The remaining five (6%) HIV antibody-positive hemophiliacs were known to be positive in 1981 (one), 1983 (one), and 1985 (three); however, the last negative sample was either 3 or more years prior or unavailable, and thus these five patients were not included in seroconversion determination. Four were asymptomatic and one had ARC. The group of 79 seropositive hemophiliacs was selected solely on the basis of seroposi-

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tivity (HIV antibody positivity) and availability of serial samples; they did not differ from the other HIV seropositive hemophiliacs in age, race, or blood product usage. Further, only one sample per year per patient was used to determine seroconversion. When more than one sample was available, the latest available sample in a given year was the one used for determining seroconversion. Because dates of seroconversion were determined by yearly samples, they may underestimate the date of seroconversion by 6 months or more.

Viral antibody tests. Antibody (IgG) to HIV was measured by an enzyme-linked immunosorbent assay (ELISA) technique using inactivated HTLV-III antigen from the H9/HTLV-III B cell line (Abbott Laboratories, Chicago) or using inactivated whole LAV antigen growth in the CEM-F cell line (Genetic Systems, Seattle). All samples with positive HIV antibody tests were subjected to Western blot confirmation performed at Abbott Laboratories or at Genetic Systems by standard techniques.⁸

Helper/suppressor ratios. T lymphocytes, including T helper and T suppressor cells, were determined on a Spectrum III Cytofluorogram, using OKT₃, OKT₄, and OKT₈ antibodies (Ortho Diagnostic Systems, Inc, Raritan, NJ).⁹

T colony assay. The T colony assay was performed by techniques previously described.^{10,11} Mononuclear cells were isolated from heparinized samples with Ficoll-hypaque and washed in Hank's balanced salt solution (HBSS). They were then incubated in RPMI 1640 media, containing 10% pooled human AB serum, with phytohemagglutinin (PHA) 5 μ L per plate, in 35-mm gridded plastic petri dishes, for seven days in a 5% CO₂, 37°C humidified atmosphere (PHA, Wellcome Research Laboratories, Research Triangle Park, NC). Colonies were enumerated with an inverted microscope, with tight clusters of at least 25 cells scored as colonies. The same technique was repeated with the addition of interleukin-2 (IL-2) (Electronucleonics Laboratories, Silver Spring, MD), at a concentration of 5% per plate. Simultaneously performed controls consisted of 17 healthy heterosexual individuals.

Statistics. The T₄ number of hemophiliacs with and without AIDS was compared by chi-square analysis.

RESULTS

We previously reported, on the basis of ELISA testing, that 80 of 190 hemophiliacs were HIV antibody-positive in 1984.⁴ Subsequent analysis of these sera by Western blotting indicated that nine of these were false-positive; as such, the adjusted prevalence of antibody positivity was 37.4% (71 of 190). These nine included one treated with factor VIII concentrate, one treated with factor IX concentrate, and seven treated with cryoprecipitate. Of the 181 available for repeat study in 1986 (nine moved), a total of 82 were HIV antibody-positive (all Western blot confirmed), for a prevalence of 45.3%. The HIV seropositive group included 50% (74 of 149) of patients with hemophilia A, of whom 95% were severe (<0.01 U/mL factor VIII) and 5% were moderate (0.01 to 0.05 U/mL), and 25% (eight of 32) of patients with hemophilia B, of whom 88% were severe and 12% moderate. By treatment (Table 1), the HIV seropositive included 82% (63 of 77) of those receiving factor VIII concentrate, of whom 97% were severe and 3% moderate; 48% (13 of 27) of those receiving factor IX concentrate, of whom 92% were severe and 8% moderate; 10% (six of 60) of those receiving cryoprecipitate, of whom 67% were severe and 33% mild; and none of those receiving fresh frozen plasma.

Ten new HIV seroconversions occurred between 1984 and

Table 1. Comparison of HIV Antibody Seroprevalence in a Hemophilic Cohort in 1984 and 1986

Blood Product	No. HIV Ab(+)/ No. Tested			
	1984* (n = 190)		1986 (n = 181)	
FVIII	60/82	(73.2%)	63/77	(81.8%)
FIX	8/30	(26.7%)	13/27	(48.1%)
CRYO	3/59	(5.1%)	6/60	(10.0%)
FFP	0/19	(0%)	0/17	(0%)
	71/190	(37.4%)	82/181	(45.3%)

Abbreviations: FVIII, factor VIII concentrate; FIX, factor IX concentrate; CRYO, cryoprecipitate; FFP, fresh frozen plasma.

*Revised for one FVIII-treated, one FIX-treated, and seven Cryo-treated who were originally HIV antibody-positive (1984), but on subsequent testing were found to be Western blot negative.

1986 (in addition to one 1984 seroconversion detected in a previous study).⁴ These included three treated with factor VIII concentrate, five treated with factor IX concentrate, and two treated with cryoprecipitate. These occurred before switching to heat-treated products in all but one who was the recipient of an unscreened, heat-treated lot of factor VIII concentrate subsequently recalled. In addition, five other patients, on whom serial samples were unavailable, were found to be HIV-seropositive: one in 1981, one in 1983, and three in 1985. Thus, by 1986, there was an 11.7% increase in HIV seroprevalence in those treated with factor VIII concentrate, an 80.1% increase in those treated with factor IX concentrate, and a 96.1% increase in those treated with cryoprecipitate, as compared with 1984 figures. Of the 16 HIV-seronegative hemophiliacs (13 cryoprecipitate-treated; three fresh frozen-plasma treated) who have received solely heat-treated, HIV antibody-screened concentrates since 1985, none have seroconverted (12 or more months follow-up).

Based on seroconversion data on 77 serially sampled HIV antibody-positive hemophiliacs (1977 through 1986), the earliest seroconversion occurred in 1978, and the peak occurred in 1982 (Table 2). Half or more of those treated with factor VIII concentrate who seroconverted did so in 1982 (44 of 60), whereas half or more of those treated with factor IX concentrate (six of 12) or cryoprecipitate (three of five) who seroconverted, did so in 1983.

When the CDC HIV classification system⁷ was applied to these 79 HIV seropositive hemophiliacs, 35% were class IV, 12% were class III, and 53% were class II (Table 3). Of those seropositive 6 or more years (ie, seroconverting in 1978, 1979, 1980), half have developed AIDS; the cumulative AIDS incidence for those seropositive 5 or more years is 32% (Table 3). The overall AIDS incidence based on all seropositive hemophiliacs in this cohort (n = 82) is 12% (ten of 82) and, based on all hemophiliacs (seropositive and seronegative) in this cohort (n = 181), is 5.5% (ten of 181).

The immunologic changes before, during, and after HIV seroconversion in 63 HIV antibody-positive hemophiliacs, on whom the date of seroconversion was known and who were studied in 1983, 1985, and/or 1986, are shown in Fig 1. Before HIV exposure, the absolute T₄ lymphocyte number

Table 2. HIV Seroconversion Rate and Blood Product Treatment in 77 HIV Antibody-Positive Hemophiliacs

Year of Seroconversion	No. Becoming Ab(+) / No. remaining Ab(-)	Blood Product Treatment		
		FVIII	FIX	CRYO
1977	0/77 (0%)	—	—	—
1978	2/77 (2.6%)	1	1	0
1979	2/75 (2.7%)	2	0	0
1980	2/73 (2.7%)	2	0	0
1981	13/71 (18.3%)	13	0	0
1982	28/58 (48.3%)	26	1	1
1983	19/30 (63.3%)	13	4	2
1984	3/11 (27.3%)	1	1	1
1985	7/8 (87.5%)	1	5	1
1986	1/1 (100%)	1	0	0
		60 (78%)	12 (16%)	5 (6%)

The five HIV antibody-positive hemophiliacs who were not included in this table because their date of seroconversion was not known included three receiving FVIII (HIV Ab(+)) in 1981, 1985, 1985), one receiving FIX (HIV Ab(+)) in 1983), and one receiving cryo (HIV Ab(+)) in 1985) (see text).

was lower than control; at seroconversion, the T_4 number remained unchanged, while the T_8 lymphocyte absolute number increased, and T cell function (T colony assay) declined. Over the 5-year period following seroconversion, a gradual decrease in the absolute number of T_4 lymphocytes was noted, reaching a mean of $135 \pm 26/\text{mm}^3$ at 5 years after seroconversion. The T_4 helper number fell below $100/\text{mm}^3$ in 16 of 63 HIV seropositive hemophiliacs studied. Of these 16, ten (62.5%) developed AIDS, while none of 47 (0%) with a T helper number above $100/\text{mm}^3$ had AIDS ($X^2 = 28.45$, $P < .001$). The mean T_8 number remained in the 400 to $700/\text{mm}^3$ range in the 5-year period following seroconversion, declining to a mean of $266 \pm 77/\text{mm}^3$ (range, 11 to $764/\text{mm}^3$) in eight hemophiliacs at the time of diagnosis of AIDS. The T_4/T_8 ratio declined from a mean pre-HIV exposure value of 1.38 ± 0.23 to 0.42 ± 0.48 at 5 years postseroconversion, reaching 0.21 ± 0.08 in eight hemophiliacs at diagnosis of AIDS.

A marked decline in T lymphocyte function, measured by T colony growth in the T colony assay, was observed following seroconversion, reaching 1193 ± 537 colony counts by 5 years after seroconversion, and 377 ± 231 colony counts in eight patients at diagnosis of AIDS.

DISCUSSION

This study indicates that the overall prevalence of HIV antibody in hemophiliacs has increased significantly since 1984, with 82% of factor VIII concentrate-treated, 48% of factor IX concentrate-treated, and 10% of cryoprecipitate-treated patients now HIV seropositive. The small number of hemophiliacs who have recently (since mid-1984) seroconverted (11 in this center) occurred before their being switched to screened, heat-treated concentrates or screened single-donor products. Further, none of the 16 previously HIV-seronegative hemophiliacs who have now received solely screened, heat-treated concentrates at this center since 1985 (12 or more months follow-up) have seroconverted. These data suggest that, although new seroconversions have continued to occur since 1984, future exposure to HIV in hemophiliacs is declining and should disappear as a result of HIV antibody screening of donors and heat-treatment of blood products. Thus, hemophiliacs who are HIV antibody-negative would appear to have very minimal risk of future HIV exposure.

The 5.5% AIDS incidence in this hemophiliac cohort (including both HIV-seropositive and HIV-seronegative) is almost four times higher than the national average of 1.5% (303 cases per 20,000 individuals with hemophilia).² The reason for this higher incidence in western Pennsylvania is unknown, although not inconsistent with previously described geographic differences in hemophilia AIDS incidence in the United States.³ The hemophilia AIDS incidence may relate to date of seroconversion, and comparison of peak seroconversion date among hemophilia centers might be extremely helpful in resolving this issue.

The projection for new cases of AIDS among HIV-seropositive hemophiliacs, based on data from this study, is

Table 3. Clinical Findings and AIDS Incidence in 77 HIV Antibody-Positive Hemophiliacs by Year of Seroconversion

Year of Seroconversion	No. Seroconverting	Clinical Classification					AIDS Incidence by Year of Seroconversion†	Cumulative AIDS Incidence Rate	Duration of HIV Infection (yr)
		Class II Asymptomatic	Class III Lymphadenopathy	Class IV					
				Other*	ARC	AIDS			
1978	2	1	0	0	0	0	1/2 (50%)	1/2 (50%)	8
1979	2	0	0	0	1	1	1/2 (50%)	2/4 (50%)	7
1980	2	1	0	0	0	1	1/2 (50%)	3/6 (50%)	6
1981	13	7	1	1	1	3	3/13 (23%)	6/19 (32%)	5
1982	28	15	3	3	5	2	2/28 (7%)	8/47 (17%)	4
1983	19	12	3	1	1	2	2/19 (11%)	10/66 (15%)	3
1984	3	2	1	0	0	0	0/3 (0%)	10/69 (14%)	2
1985	7	2	1	0	4	0	0/7 (0%)	10/76 (13%)	1
1986	1	1	0	0	0	0	0/1 (0%)	10/77 (13%)	<1
	77	41	9	5	12	10			
		(53%)	(12%)	(6%)	(16%)	(13%)			

Of the five patients whose seroconversion dates are unknown and thus not included in this table, four were asymptomatic (class II) and one had ARC (class IV).

*Includes neurologic symptomatology, herpes zoster, and oral candida infection.

†As of December 1986.

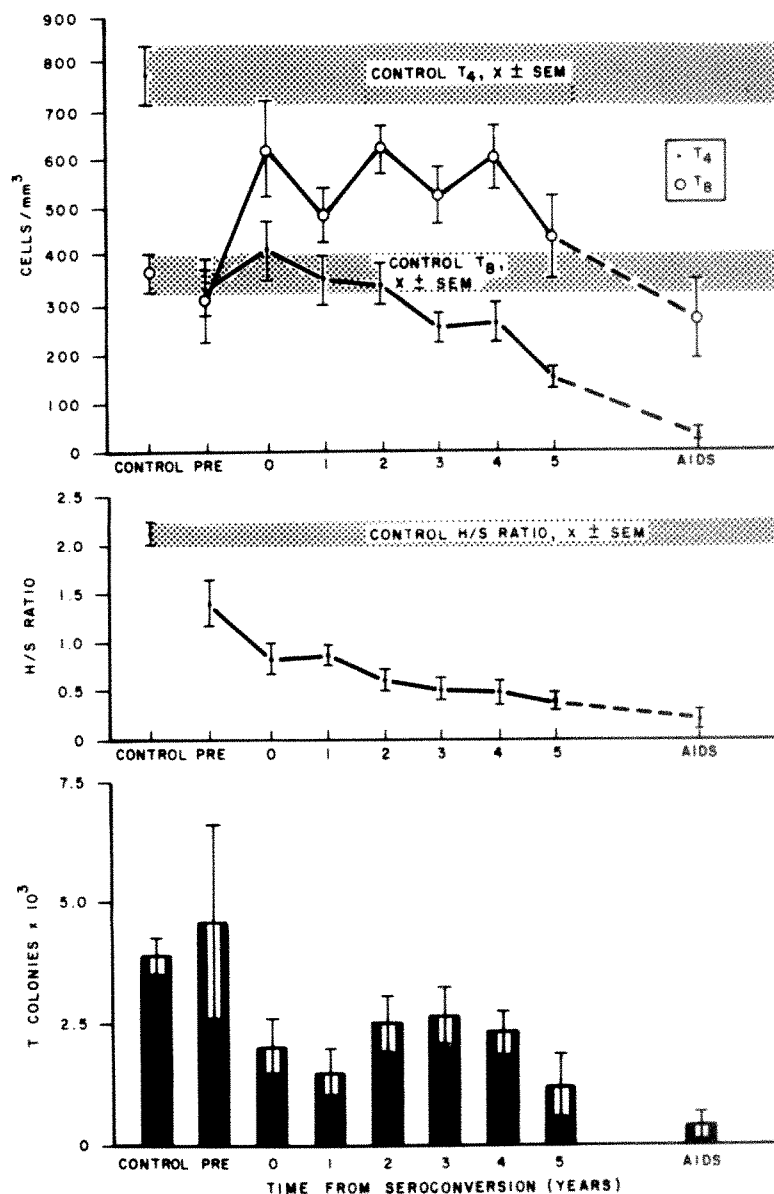


Fig 1. T lymphocyte subsets and T colony assay in HIV antibody-positive hemophiliacs before, during, and after seroconversion. A total of 63 hemophiliacs on whom the date of seroconversion was known were evaluated in 1983, 1985, and/or 1986. Each data point is expressed as mean $\pm$ SEM. Results are expressed as mean T_4 helper lymphocyte/mm³ (●), mean T_8 suppressor lymphocyte/mm³ (○), and mean T colony counts (colonies $\times 10^3$) (■) (see text). The X axis represents time from seroconversion in years. Control values are shown to the left of the hemophilia data. The eight patients in the AIDS category developed AIDS 3 to 6 years following seroconversion, and data for this group were taken at the time AIDS was diagnosed, rather than a specific time interval from seroconversion.

that in those seropositive for 5 years or more, the AIDS incidence is 32%. These data coincide with AIDS attack rates for other high-risk groups,^{6,12} but contrast with the trend reported by a recent CDC survey² showing a slowing in the rate of hemophilia-associated AIDS over the last five quarters since mid-1985. In interpreting these data, it is important to note the small number of patients in the denominator (19) and the imprecision in exact time of seroconversion. However, because the peak in HIV seroconversion in hemophiliacs in this center and elsewhere occurred in 1982 and 1983,^{4,5} and because the median latency from infection (seroconversion) to development of AIDS is 5 years or longer, the number of new AIDS cases in hemophiliacs in 1987 or 1988 or later should help resolve this issue.

HIV exposure in hemophiliacs appears to result in a progressive depression of the cellular immune system, with a decline in T lymphocyte function as measured by the T

colony assay, and a decline in T_4 helper lymphocyte number, with increasing time from seroconversion. Similar defects have been described in other high-risk groups.^{13,14} Of note in this group, a T_4 helper number of less than 100/mm³ was highly predictive of AIDS, with over 60% of those falling below 100/mm³ and none of those remaining above 100/mm³ developing AIDS. It is of interest that before HIV exposure in hemophiliacs, both T_4 lymphocyte number and T_4/T_8 ratio are markedly decreased below control (at a time when T_8 is normal). These abnormalities are typical of immunologic alterations previously observed in multitransfused patients.¹⁷⁻²⁰ The causes of these abnormalities are not known, but may be related to chronic exposure to alloantigenic transfused blood proteins or exposure to infectious agents such as cytomegalovirus, hepatitis B, or non-A, non-B hepatitis. Whether these immunologic alterations predispose hemophiliacs to the development of AIDS is unknown.

Following seroconversion, the number of T₈ suppressor lymphocytes remains normal or elevates slightly following seroconversion, consistent with previous reported findings in homosexual men.²¹ Thus, an increase in absolute number of T₈ suppressor cells may be another, possibly early, marker of HIV infection.

In summary, the results of this study imply that the AIDS incidence in hemophiliacs is increasing with increasing duration of seropositivity, consistent with previous predictions for other risk groups.⁶ Extrapolation from the results of this study, however, should be tempered by the well-described geographic differences in hemophilia-associated AIDS incidence,³ which may suggest other cofactors in the development of AIDS in this group. These findings further confirm that hemophiliacs were exposed through clotting factor concentrates to infectious viruses, and not just viral antigens. A decline in T lymphocyte function, as measured by the T colony assay, and T₄ number appear to be markers of the immune defect of HIV infection. The pattern of progressive decline in T₄ lymphocytes in HIV-seropositive hemophiliacs over time suggests that, in those in whom the T helper number decreases below 100/mm³, there is a high incidence of AIDS.

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Biotinylated Erythrocytes: In Vivo Survival and In Vitro Recovery

By Takashige Suzuki and George L. Dale

Rabbit erythrocytes were biotinylated by reaction with N-hydroxysuccinimidobiotin; the average level of biotinylation was 25,000 molecules per erythrocyte. These biotinylated cells exhibited a normal survival rate when reinfused into rabbits. Two studies demonstrated that the biotin label was stable in vivo. The first was a double-labeling experiment where the biotinylated erythrocytes were also labeled with ^{14}C -cyanate; on reinfusion, the loss of biotinylated erythrocytes and ^{14}C -cyanate label occurred in uni-

son. The second study demonstrated that biotinylated erythrocytes that had been reinfused into rabbits could later be selectively isolated by attachment to an avidin support. This technique will facilitate a variety of studies that require the ability to label a specific cohort of cells in vitro and then reisolate those same cells after in vivo recirculation.

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THE MECHANISMS that govern erythrocyte senescence have been extensively studied, and numerous hypotheses have been proposed¹⁻⁷; however, definitive studies have been hindered by the lack of techniques that allow isolation of well-defined age groups of erythrocytes. Generally the isolation of specific erythrocyte subpopulations has relied on physical techniques based on putative changes in aged erythrocytes such as density or volume alterations.^{8,9} However, the ability of existing physical techniques to give unequivocal results when trying to isolate a defined age group of erythrocytes has been questioned.^{10,11}

A number of other procedures for the isolation of aged erythrocytes have been reported. One interesting technique allows the isolation of senescent rat erythrocytes.¹² In this procedure, a large number of starting animals are used. At frequent intervals, a fraction of the group is killed to provide blood to hypertransfuse the remaining rats. The steadily decreasing number of surviving rats will not synthesize new erythrocytes while they are polycythemic from the transfusions, resulting in a few animals at the end of the experiment having "aged erythrocytes." While this technique is quite effective, it is also labor-intensive and consumptive of experimental animals. Another procedure that is very preliminary would isolate aged erythrocytes on the basis of surface immunoglobulin levels,^{7,13} with the assumption that aged erythrocytes have increased levels of bound immunoglobulin.

Another relevant technique is found in the work of Allison and Burn.¹⁴ These investigators transfused blood group O erythrocytes into normal blood group A volunteers; the mismatched cells were then selectively recovered by agglutination of the A erythrocytes. The technique allowed the isolation of erythrocytes up to 102 days after transfusion, but the utility of the procedure was hindered by the limitations of the differential agglutination step.¹¹

In this report, we describe a novel approach to the isolation of specific subpopulations of rabbit erythrocytes. This is achieved by the biotinylation of erythrocytes, demonstration that these biotinylated cells have a normal in vivo survival in the rabbit, and specific in vitro recovery of the biotinylated erythrocytes. This technique, therefore, allows the specific tagging of erythrocytes with a biocompatible label along with the ability to recover this labeled subpopulation after reinfusion into a rabbit.

MATERIALS AND METHODS

Polystyrene beads (200 to 400 mesh, 12% cross-linked, No. 4023) were purchased from Polysciences, Inc., Warrington, PA. Avidin,

biotin, collagenase (type IV, 360 U/mg), bovine serum albumin (BSA), 2-(4'-hydroxyazobenzene)-benzoic acid (HABA), and succinic anhydride were obtained from Sigma Chemical Co., St. Louis. N-Hydroxysuccinimidobiotin (NHS-biotin) was from Pierce Chemical Co., Rockford, IL, and gelatin was from Difco Laboratories, Detroit. N,N-Dimethyl-formamide (DMF) was purchased from Eastman Kodak Co., Rochester, NY. ^{14}C -potassium cyanate was obtained from Amersham, Arlington Heights, IL. All other chemicals were of reagent grade or better.

Synthesis of Reagents

Succinylated BSA was synthesized by the method of Chu et al.¹⁵ with slight modifications. One gram of BSA was dissolved in 100 mL of 0.5 M NaHCO_3 , pH 8.0. Succinic anhydride, 800 mg, was added as 200-mg aliquots to the BSA solution over 60 minutes at room temperature, and the pH maintained at 8.0 with NaOH. The product was dialyzed against three changes of 2 L of 10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.5 (PBS) at 4°C. Succinylated BSA was stored at a protein concentration of 8.3 mg/mL or 120 $\mu\text{mol/L}$.

Biotinylated gelatin was synthesized by the following procedure: Gelatin (60 mg) was dissolved in 10 mL of 50 mmol/L sodium borate, pH 8.0, with mild heating. After cooling, NHS-biotin (4 mg in 100 μL DMF) was added, and the mixture was incubated at room temperature for 60 minutes. The biotinylated gelatin solution (Biotin-Gelatin) was dialyzed against two changes of 1 L of PBS for four hours at room temperature, and the solution was stored at -20°C.

Spectrophotometric determination of biotin bound to gelatin: Biotin molecules bound to gelatin were determined by the method of Green.¹⁶ The HABA dye binds weakly to avidin with a shift in the dye's spectral properties; biotin easily displaces the HABA with a concomitant change in the visible spectrum. An avidin-HABA complex consisting of 0.2 mg avidin/mL of 0.25 mmol/L HABA, 0.1 mol/L sodium phosphate buffer, pH 7.0, was prepared. Different amounts of biotinylated gelatin (10 to 120 μg) were added to 2 mL of avidin-HABA complex, and their optical densities were measured at 500 nm. The biotin concentration was calculated as reported¹⁶ and found to average 94 nmol biotin/mg gelatin.

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Biotinylation of Erythrocytes

Twenty milliliters of blood obtained from a male rabbit (New Zealand White, 2 to 3 kg) by auris arteriopuncture was collected into a heparinized tube. The blood was washed in PBS three times, and the buffy coat was removed. The cells were suspended to a 10% hematocrit in PBS containing 15 mmol/L glucose. Ten milliliters of suspension was added to 15 μ Ci ^{14} C-potassium cyanate, 0.83 μ Ci/ μ mol,¹⁷ and the mixture was incubated at 37°C for 30 minutes in a shaking bath. Succinylated BSA, 295 nmol, was then added to the cell suspension and gently shaken for three minutes at room temperature. Finally, 295 nmol NHS-biotin (100 μ g, stock solution 2 mg/mL of DMF) was added to the mixture, and the incubation was continued at 37°C for 60 minutes. The mean substitution level with this recipe was $26,600 \pm 7,300$ ($n = 6$) biotin molecules per erythrocyte.

The ^{14} C-labeled and biotinylated erythrocytes were washed three times in PBS and resuspended to a 50% hematocrit. To examine cell shape, a portion of the cells was fixed in 1% glutaraldehyde in PBS; the absence of abnormal cells (echinocytes and elliptocytes) was confirmed by light microscopy. The biotin and ^{14} C content of the cells was determined as described below. The remaining erythrocyte suspension was reinfused by auris venipuncture into the same rabbit from which the original blood was obtained. At five minutes after reinfusion, 2 mL of blood was obtained as a $T = 0$ sample by auris arteriopuncture of the opposite ear used for reinfusion. Blood samples were taken at regular intervals for up to 60 days.

Quantitation of ^{14} C and Biotin Content of Erythrocytes

The collected blood was filtered through cellulose to remove leukocytes and platelets.¹⁸ Erythrocytes were washed three times with PBS and left at approximately 20% hematocrit; an accurate hematocrit value of the resuspended sample was determined with a micro hematocrit centrifuge. The ^{14} C radioactivity level was measured as follows: The erythrocyte suspension (usually 200 to 400 μ L) was mixed with 1 mL of 10% sodium dodecylsulfate in a glass scintillation vial and shaken at 37°C. After 15 minutes, 65 μ L of 30% hydrogen peroxide was added to bleach the hemoglobin-rich solution and the incubation continued at 37°C for 30 minutes. Finally, 50 μ L of neat β -mercaptoethanol was added to neutralize any residual peroxide with a final incubation at 37°C for 30 minutes. After adding 10 mL of scintillation fluid (Safety Solve) to the colorless mixture, the samples were left at room temperature for at least two hours before counting to allow chemiluminescence to decay.

Biotin bound to erythrocytes was quantitated indirectly by saturation binding of ^{125} I-avidin. Three and one-half milliliters of washed cell suspension (20% hematocrit) in PBS were added to a 25-mL Erlenmeyer flask along with 3 mL PBS containing 1 mg/mL BSA. Each flask received 50 μ g of ^{125} I-avidin (approximately 1,000 cpm/ μ g). A background level of nonspecific ^{125} I-avidin binding to erythrocytes was determined by carrying out the incubation in the presence of 0.5 mmol/L biotin to overwhelm the small amount of erythrocyte-bound biotin. The mixture of erythrocytes and ^{125} I-avidin was incubated with shaking at 37°C for 60 minutes. The cells were washed three times with PBS and cell-bound ^{125} I radioactivity was determined.

The avidin concentration (A), in μ g avidin/mL RBC, was calculated as follows:

$$A = (B_1 - B_2)/CR$$

where B_1 is the radioactivity (cpm) of the sample without biotin, B_2 is the radioactivity of the sample with 0.5 mmol/L biotin, C is the specific radioactivity of the ^{125} I-avidin (cpm/ μ g), and R is the packed erythrocyte volume present in the assay. The number of

avidin molecules (N per erythrocyte), which is used as an estimate of biotin molecules on a cell, was calculated as follows:

$$N = (A \times 10^6 \times 6.02 \times 10^{23}) / 68,000 \times 10^{10}$$

where the molecular weight of avidin is 68,000 g/mol, Avogadro's number is 6.02×10^{23} molecules/mol, and the number of erythrocytes in 1 mL of packed cells is estimated as 10^{10} .

In Vitro Recovery of Biotinylated Erythrocytes

Preparation of avidin-biotin gelatin coated beads. Four milligrams of biotin-gelatin and 2 mL of PBS were added to 500-mg polystyrene beads in a polypropylene tube (17 $\times$ 100 mm, Falcon 2059) and mixed on a rotator for 60 minutes. The beads with bound biotin-gelatin (biotin-gelatin-beads) were washed five times with PBS by gentle centrifuging at 300 g for five seconds.

Avidin (750 μ g) and 2 mL PBS were added to the washed biotin-gelatin-beads and rotated at room temperature for 20 minutes. The beads were washed five times with PBS by gentle centrifuging at 300 g for five seconds. This final preparation is referred to as avidin-biotin-gelatin-beads.

Recovery of biotinylated cells. Biotinylated erythrocytes were withdrawn from rabbits, freed of leukocytes, and washed as detailed above. One milliliter of these erythrocytes (approximately 80% hematocrit) was mixed with 500 mg avidin-biotin-gelatin-beads in a 50-mL glass beaker and incubated on a rocking platform at room temperature for 60 minutes. The beads were transferred into a 15-mL plastic tube and washed with PBS by gentle centrifuging at 300 g for five seconds; the washing was repeated until the supernatant was clear.

To collect intact cells from the beads, collagenase (900 U in 10 mL PBS) was added to the beads with bound biotinylated erythrocytes in a 25-mL Erlenmeyer flask and the mixture incubated at 37°C for fifteen minutes. The mixture was centrifuged briefly twice at 300 g for five seconds to remove beads, and the supernatant containing biotinylated erythrocytes was taken.

RESULTS

Rabbit erythrocytes were reacted with NHS-biotin to tag the extracellular surface with covalently attached biotin. These biotinylated erythrocytes were then reinfused into normal rabbits and could be subsequently recovered by cell affinity chromatography on an avidin support. Figure 1 depicts the overall experimental scheme.

Figure 2 shows the number of biotin molecules bound to the erythrocytes as a function of the NHS-biotin concentration. Early experiments indicated that the erythrocytes needed approximately 15,000 biotin molecules per cell if the cells were to be successfully recovered by attachment to an avidin support (data not shown). Therefore, erythrocytes with an average of 25,000 to 30,000 biotin molecules per cell were used for most experiments to ensure that the cells could be recovered in vitro, as outlined below.

Rabbit erythrocytes containing an average of either 13,400 or 27,000 biotin molecules per cell and labeled with ^{14} C-cyanate were reinfused into normal rabbits. Subsequently, samples were taken from the rabbits at regular intervals for 53 days; the reported mean life span of rabbit erythrocytes is approximately 60 days.¹⁹ The data in Fig 3 demonstrate that the biotinylated and ^{14} C-labeled erythrocytes had a viability indistinguishable from that of control ^{14} C-labeled erythrocytes.



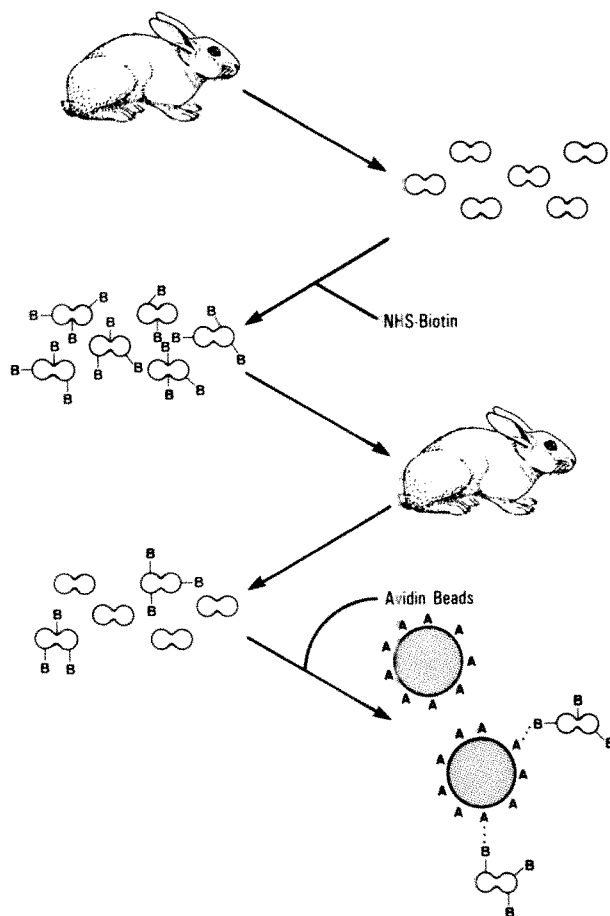


Fig 1. Overall scheme for labeling and recovery of rabbit erythrocytes. Erythrocytes were biotinylated (B) by reaction with NHS-biotin; these biotinylated cells could be selectively recovered by attachment to avidin (A) coated polystyrene beads.

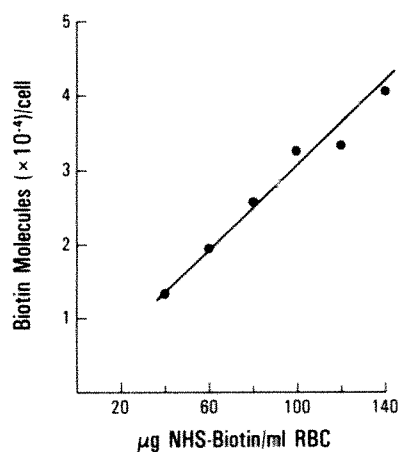


Fig 2. Derivatization of erythrocytes with NHS-biotin. Rabbit erythrocytes in PBS were reacted with various concentrations of NHS-biotin. The number of biotin molecules bound per erythrocyte was determined by saturation binding of ^{125}I -avidin to the cells (see Materials and Methods section for details).

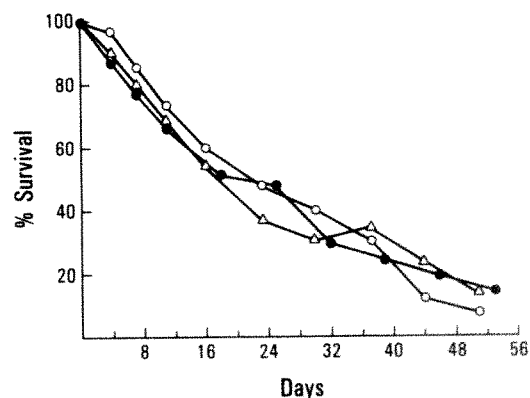


Fig 3. In vivo survival of biotinylated and control erythrocytes. Blood was drawn from three rabbits; two of the preparations were biotinylated (open circles, 27,000 biotin molecules per cell; triangles, 13,400 biotin molecules per cell) and the third served as a control (closed circles). All three preparations were labeled with ^{14}C -cyanate and reinfused into the respective donor rabbits. Samples were drawn at regular intervals and the ^{14}C remaining expressed as percent of starting level ($T = 5$ minutes sample).

Figure 4 verifies that the biotin present on the erythrocytes is stable in vivo. For these experiments it was necessary to biotinylate the erythrocytes more heavily (126,000 biotin molecules per cell) in order to measure the actual level of erythrocyte-bound biotin after reinfusion; this increased level of biotinylation results in a moderately decreased in vivo life span for these cells. As shown in Fig 4, the level of erythrocyte-associated ^{14}C -cyanate and biotin present in the rabbits decreased in parallel, indicating that the biotin is stable on the erythrocytes in vivo. This stability of the biotin tag is particularly significant in that serum contains biotinidase, an enzyme capable of cleaving biotin from some peptide substrates.²⁰ While biotinidase is reported to be active only with low molecular weight substrates, the possi-

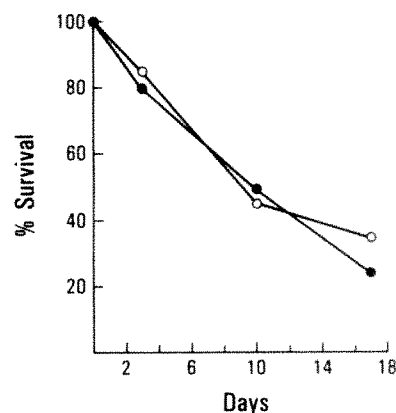


Fig 4. Co-survival in vivo of biotin and cyanate labels on rabbit erythrocytes. Erythrocytes were labeled with ^{14}C -cyanate and heavily with NHS-biotin to a level of 126,000 biotin molecules per cell. These cells were reinfused, and the level of the two labels (^{14}C -cyanate, closed circles; biotin, open circles) was monitored for 17 days (see Materials and Methods section for details). The percent survival of each label was calculated based on the $T = 5$ minutes sample.

bility that prolonged exposure of biotinylated erythrocytes to biotinidase might result in the enzymatic removal of some biotin could not be excluded *a priori*.

The *in vitro* recovery of biotinylated erythrocytes was first examined using synthetic mixtures of 95% control and 5% biotinylated erythrocytes to mimic the approximate ratio expected in later experiments. The biotinylated erythrocytes were also labeled with ^{51}Cr -sodium chromate, which served as a marker for the efficiency and accuracy of the *in vitro* recovery system. Table 1 shows the data from a representative experiment. The efficiency of recovery of biotinylated erythrocytes varied from 25% to 50% using the conditions described in the Materials and Methods section. More important than the efficiency of recovery is its accuracy, i.e., how much contamination is there by nonbiotinylated erythrocytes? This was examined by determining the ratio of radioactivity to hemoglobin (Hb) in the recovered cells. If nonbiotinylated and nonradioactive control cells are bound to the avidin-biotin-gelatin-beads, the cpm/Hb ratio would be dramatically altered. As shown in Table 1, the recovered cells have a cpm/Hb ratio that is approximately 90% of the specific activity of the pure, biotinylated erythrocyte population.

The recovery of biotinylated erythrocytes after reinfusion into rabbits is shown in Table 2. As discussed above, the specific recovery of ^{14}C -labeled and biotinylated erythrocytes is indicated by the constant ^{14}C /Hb ratio over time. Also shown are the ^{14}C /Hb ratios for the unfractionated samples before attachment to the avidin-biotin-gelatin-beads. As expected, this latter ratio drops as the biotinylated cells are progressively removed from circulation; however, the ^{14}C /Hb ratios for the recovered cells remain constant, again indicating the selectivity of the *in vitro* recovery.

Intact, biotinylated erythrocytes could be recovered from the avidin-biotin-gelatin-beads by incubation with collagenase; the collagenase enzymatically degrades the gelatin bound to the polystyrene beads, thereby disrupting the anchor that holds the biotinylated erythrocytes to the beads. The yield of intact cells varied between 33% and 66% of the

Table 1. Model Studies on Recovery of Biotinylated Erythrocytes

Sample	Recovery of Biotinylated Erythrocytes		Specific Activity of Recovered Cells	
	Bound to Beads* (%)	Recovered After Collagenase Treatment of Erythrocyte/Beads*	cpm/mg Hb†	Control (%)
A	32.4	13.0	1605	91.1
B	29.0	14.5	1595	90.5
C	31.0	15.2	1520	86.2

A synthetic mixture of 95% control erythrocytes and 5% biotinylated and ^{51}Cr -labeled erythrocytes was mixed with avidin-biotin-gelatin-beads as described in Materials and Methods.

*Recovery as percent of total radioactivity of biotinylated erythrocytes before attachment to the avidin-biotin-gelatin-beads.

†Specific activity of starting biotinylated erythrocyte population was 1760 cpm/mg Hb, and the specific activity of the synthetic mixture of 95% control and 5% biotinylated, ^{51}Cr -labeled erythrocytes was 75.3 cpm/mg Hb.

Table 2. Recovery of Biotinylated Erythrocytes After *In Vivo* Circulation

Rabbit	Sample*	Days after Infusion			
		0	7	18	28
1	Unfractionated cells	164	135	41.9	32.5
	Biotinylated cells	741	599	724	719
2	Unfractionated cells	160	125	60.5	30.9
	Biotinylated cells	673	566	591	674
3	Unfractionated cells	135	155	66.5	17.0
	Biotinylated cells	932	685	531	593

Values expressed as cpm of ^{14}C -cyanate per milligram hemoglobin. The degree of biotin substitution for each rabbit, expressed as biotin molecules per erythrocyte, was 16,900 (rabbit 1), 30,200 (rabbit 2), and 68,200 (rabbit 3).

*Unfractionated sample represents entire erythrocyte population, while biotinylated cells refers to the erythrocytes recovered by affinity isolation with avidin-biotin-gelatin-beads.

bound cells; this large variability is not understood and is currently under investigation.

DISCUSSION

This report describes a technique for the biotinylation of rabbit erythrocytes that does not affect the *in vivo* survival of the derivatized cells but still allows for the specific isolation of the biotinylated erythrocytes by an avidin support. Figure 1 shows the general experimental scheme.

The level of biotinylation is quite low, with an average of 26,600 biotin molecules per erythrocyte. However, preliminary experiments revealed that the erythrocyte morphology was altered by the NHS-biotin incubation. Greater than 90% of the cells after the derivatization were echinocytes rather than normal biconcave discs, although an almost normal shape could be restored to the cells by repeated washings in the presence of BSA. We subsequently found that inclusion of succinylated BSA, equimolar to that of NHS-biotin, prevented the shape change and resulted in a cell morphology for the biotinylated erythrocytes that was indistinguishable from the control cells. The succinylated BSA serves as a nonnucleophilic carrier for the hydrophobic NHS-biotin. In the presence of this carrier, the NHS-biotin is presumably prevented from intercalating into the erythrocyte membrane and causing a reversible shape change similar to that observed with several lipophilic drugs.²¹

As detailed in the Results section, the biotinylated erythrocytes had an *in vivo* survival indistinguishable from that of control cells. One initial concern with this technique was that the rabbit might produce an immune response against the biotinylated cells. Clearly the data in Fig 3 would suggest that this is not a major concern, since antibody produced during the 53 days that biotinylated erythrocytes were present would be expected to affect the cell's survival. However, additional experiments are in progress to systematically examine the question of whether an immune response occurs.

In vitro recovery of the biotinylated erythrocytes was achieved with an avidin-biotin-gelatin-bead support. The major advantage of this support is that the gelatin anchor can be cleaved enzymatically with collagenase, thereby releasing

intact erythrocytes from the beads. It should be mentioned that the released cells still have bound avidin as well as biotin-gelatin fragments, which could potentially interfere with some analyses.

The impetus to develop this technique was an interest in erythrocyte senescence, which requires the isolation of aged erythrocytes. The utility of the procedure is shown by the following example. If a random population of rabbit erythrocytes is biotinylated and reinfused, then 50 days later the only surviving biotinylated cells will now be between 50 and 60 days old and represent those cells that were from zero to ten days old at the time of biotinylation. This procedure will, therefore, greatly facilitate the examination of putative factors related to erythrocyte senescence. However, if the biotinylated cells are isolated 30 days after reinfusion, the

biotinylated population represents cells from 30 to 60 days old, a much broader range. An additional improvement in the technique would be to initially isolate reticulocytes for biotinylation. This would allow the subsequent recovery of a narrowly defined age group of cells at any time after reinfusion.

Another potential use for this biotinylation method would be as an alternative to the currently used radioactive chromium techniques for erythrocyte survival studies. The benefits would include not exposing the test subject to radioactivity but would be partially offset by the increased handling of the cells required for biotinylation. As outlined above, before this technique can be used in humans further experimentation will be required to determine if there is an immune response to the biotinylated cells.

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Direct Evidence for the Interaction of the Nucleotide Affinity Analog 5'-p-Fluorosulfonylbenzoyl Adenosine With a Platelet ADP Receptor

By William R. Figures, L. Marie Scarce, Patricia DeFeo, Gwendolyn Stewart, Fengxin Zhou, John Chen, James Daniel, Roberta F. Colman, and Robert W. Colman

Previous reports have indicated that the nucleotide affinity analog 5'-p-fluorosulfonylbenzoyl adenosine (FSBA) at concentrations between 40 and 100 $\mu\text{mol/L}$ and at times greater than 20 minutes covalently modifies a single protein component on the external platelet membrane surface and that adenosine diphosphate (ADP) protects against this reaction. That this protein is an ADP receptor linked to platelet activation is shown by FSBA inhibition of ADP-mediated platelet shape change, aggregation, and fibrinogen receptor exposure. In this report, further evidence for the interaction of FSBA with the ADP receptor on platelets is provided by the observation that FSBA at high concentrations (100 to 500 $\mu\text{mol/L}$) behaves as a weak agonist to

produce platelet shape change within one minute as detected by spectroscopic assay and scanning electron microscopy with concomitant phosphorylation of the light chain of platelet myosin. The specificity of FSBA as an agonist is demonstrated by inhibition of FSBA-induced shape change by ATP and the covalent incorporation of SBA as well as the failure of 5'-fluorosulfonylbenzoyl guanosine (FSBG) to cause shape change. In contrast, incubation of platelets with low concentrations of [^3H]-FSBA (40 $\mu\text{mol/L}$) is not associated with stimulation of platelet shape change or myosin light chain phosphorylation.

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AN EARLY RESPONSE of human blood platelets to various agonists is a morphological change in which they are transformed from discs to spiny spheres. The latter are manifested by the appearance of cytoplasmic projections or pseudopodia. This transformation can be seen morphologically by scanning electron microscopy or optically by the decreased light transmission of stirred platelet suspensions. Adenosine diphosphate (ADP), a potent inducer of platelet shape change¹ and aggregation, possibly effects these responses of binding of the nucleotide to a cell surface receptor. The chemical identity of this receptor is unknown, although recent evidence suggests that it is distinct from an ADP receptor linked to adenylate cyclase.²

The nucleotide affinity analog, 5'-fluorosulfonylbenzoyl adenosine (5'-FSBA) has been demonstrated to modify covalently the nucleotide binding domains of more than 30

enzymes representing active and allosteric sites³ as well as the ATP binding sites of actin and myosin on the internal surface of the platelet membrane.⁴ Bennett et al⁵ have shown that FSBA is covalently incorporated into a single polypeptide (M_r 100,000), aggregin, on the external surface of the platelet membrane. The labeling of this protein demonstrates considerable specificity, since it is protected by ADP and its competitive inhibitor ATP but not by adenosine, AMP, or epinephrine. Concomitant with this modification, a rapid inhibition of ADP-induced shape change of platelets in plasma was observed. This inhibition was progressive with time of incubation with FSBA. Figures et al⁶ showed that FSBA treatment of intact washed platelets resulted in the inhibition of ADP-mediated aggregation as well as exposure by ADP of latent fibrinogen binding sites. The labeled protein is thus an attractive candidate for a nucleotide binding site that mediates shape change and aggregation as well as exposure of fibrinogen receptors in human platelets.

The designation of the FSBA-sensitive protein as the ADP shape change and aggregation receptor has been to this point based on the correlation between increased incorporation of the radiolabeled form of FSBA and enhanced inhibition of ADP-related responses of the cell. This communication presents direct evidence that FSBA is capable of interacting with an ADP receptor on the platelet cell surface.

MATERIALS AND METHODS

Materials. FSBA was prepared by the method of Colman et al.⁷ The synthesis involves condensation of adenosine with p-fluorosulfonylbenzoyl chloride (Sigma Chemical Co, St Louis). For synthesis of [^3H]FSBA, [$2\text{-}^3\text{H}$]-adenosine (New England Nuclear) was used. The radiolabeled compound had a specific radioactivity of 20 Ci/mol. FSBA concentrations were determined spectrophotometrically in ethanol using a molar extinction coefficient of 1.35×10^4 L/mol/cm⁻¹ at 259 nm. 5'-p-fluorosulfonylbenzoyl guanosine (FSBG) was synthesized by the method of Pal et al.⁸ The concentrations of FSBG were determined spectrophotometrically using an extinction coefficient of 7.3×10^3 L/mol/cm⁻¹ at 275 nm. Both compounds were stored over dessicant at -30°C and solutions in dimethyl formamide (DMF) prepared on the day of use. The final concentration of DMF used in every experiment was less than 2.0%, which had no influence on platelet shape change. Equine collagen

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fibrils were purchased from Hormon-Chemie, Munich, Germany. All other chemicals were of reagent grade or better.

Adenosine deaminase. Since FSBA can hydrolyze to adenosine, which inhibits platelet shape change, adenosine deaminase (Sigma) was added to washed platelets just before all incubations with FSBA. The concentration used (2 U/mL) is sufficient to eliminate the inhibitory action of adenosine (400 μ mol/L) on platelet shape change.

Preparation of apyrase. Potato apyrase was prepared using the method of Molnar and Lorand.⁹ These preparations contain both adenosine diphosphatase (ADPase) and adenosine triphosphatase (ATPase) activities as well as a platelet aggregating lectin. The lectin was separated from the apyrase using fetuin-agarose affinity chromatography.¹⁰

ATP purification. The disodium salt of equine muscle adenosine triphosphate (ATP) (Sigma) was dissolved in cold (4°C) deionized water. The ATP was separated from any ADP on a Dowex 50AG-X4 column in the cold. Fractions were collected in an equal volume of phosphate (0.24 mol/L) and EDTA (1×10^{-4} mol/L) buffer. The concentration of the ATP was determined by optical density $\epsilon_{258} = 15.3 \times 10^3$ L/mol/cm⁻¹.

Preparation of washed platelets. Platelet-rich plasma (PRP) was prepared from 9 vol of whole human blood and 1 vol of 3.8% sodium citrate solution by differential centrifugation ($120 \times g$, 30 minutes, 23°C). Platelets were separated from plasma by centrifugation ($1,800 \times g$, 20 minutes, 23°C). The platelet pellet was resuspended in a volume equal to the original PRP volume with a diluted Tyrode's buffer (buffer A, 4 vol of Tyrode's buffer with 2.0 mmol/L magnesium chloride diluted with 1 vol of 3.8% sodium citrate adjusted to pH 6.5 with 1 mol/L citric acid). This platelet suspension was incubated in the presence of 3 μ L apyrase/mL platelet suspension at 37°C for 20 minutes and then sedimented as with the PRP. The pellet was again resuspended in buffer A, incubated with apyrase, and sedimented. After centrifugation, the platelet pellet was resuspended to the original PRP volume with a modified Tyrode's buffer (buffer B: Tyrode's buffer with 2.0 mmol/L magnesium chloride and 2.0 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid, pH 7.4). The platelet suspension was adjusted to a platelet count of 1 to 2×10^8 /mL and kept at 23°C until used.

Measurement of platelet shape change. The washed platelet suspension (0.25 mL) was diluted with an equal volume, 5mmol/L EDTA in 0.15 mol/L NaCl, pH 7.4, to a final platelet count of 0.5 to 1.0×10^8 /mL just before adding the compounds to be tested in a

cuvette of a Chronolog Lumi-Aggregometer (Chronolog Corp, Havertown, PA). The reference channel contained suspended platelets that had been incubated with 10 μ mol/L ADP. The compounds were added in volumes of 25 to 50 μ L and the change in light transmittance recorded in a chart recorder. The decrease of light transmittance was measured, and the greatest decrease in light transmittance was defined as 100% of maximum shape change. All other measurements for a specific test compound on platelet suspension are given in relation to this value. The rate of decrease in light transmittance was measured with the slope of tracings. The highest rate of change was defined as 100% of maximum rate of shape change, and all other rates for a specific compound and platelet suspension are expressed in terms of the maximum. ATP release was also monitored simultaneously using firefly luciferase in selected experiments.

Observation of shape change by scanning electron microscopy. Platelets were prepared for scanning electron microscopy by adding 2.5% glutaraldehyde in Tyrode's buffer. The samples were fixed for 30 minutes at room temperature and kept overnight at 4°C. The next day, platelets were deposited with gentle suction (10 mm Hg) on a 1- μ m pore nucleopore membrane. The membrane and cells were then post-fixed in 1% osmium tetroxide, dehydrated with graded alcohol, dried at the critical point, and coated with gold. Photomicrographs were made at $\times 5,000$ magnification and 100 to 120 platelets from each sample were classified as discoid, spiny sphere, or intermediate forms. All samples were coded to reduce subjective bias.

Covalent incorporation of [³H]-SBA into platelet membrane protein. Previous studies have indicated that FSBA (100 μ mol/L) modifies a single polypeptide of M_r 100,000, aggregin, on the platelet membrane when intact cells were labeled as assessed by SDS polyacrylamide electrophoresis.^{4,6} Using identical methods, similar labeling of a single component was observed when FSBA (400 μ mol/L) was incubated with washed platelets for 60 minutes, but the incorporation was greater compared with an aliquot of the same suspension incubated with 100 μ mol/L [³H]-FSBA (Fig 1). Covalent modification of platelet membrane protein by [³H]-SBA was measured using a dialysis method. Washed platelets were incubated with [³H]-SBA for specific time periods. The cell suspension (1 mL) was added to a solution of dithiothreitol (DTT) (100 μ L, 2 mol/L) to stop the reaction by hydrolysis of excess FSBA. The cells were then sedimented and supernatants carefully aspirated. The pellets were then solubilized in phosphate buffer (0.04 mol/L, pH 7.2) containing SDS (2.0%), urea (8 mol/L), and EDTA (0.10 mol/L). In order to remove unbound ligand, the solutions were dialyzed in a multiple

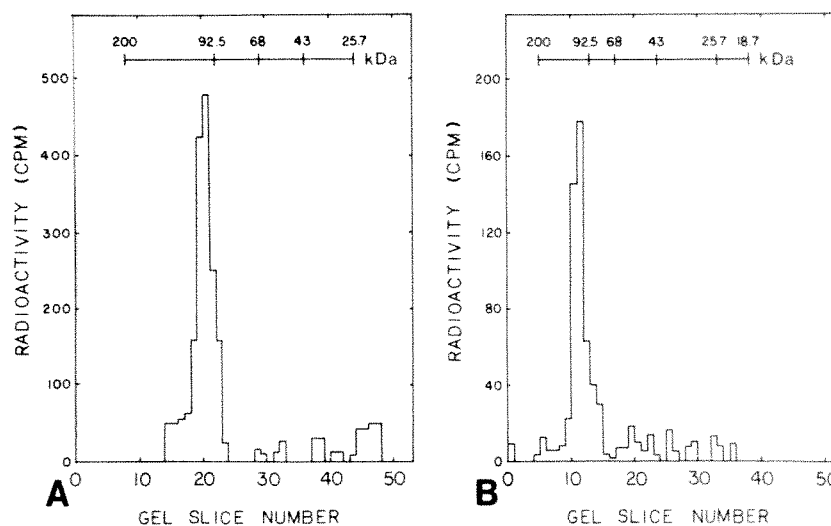


Fig 1. Incorporation of [³H]-SBA into intact platelets at 100 and 400 μ mol/L. Either [³H]-FSBA at 400 μ mol/L (A) or 100 μ M (B) was incubated with washed platelets. Extensive dialysis was performed as reported in the Materials and Methods section, and SDS gel electrophoresis and gel slicing and counting were performed as in ref. 6. Molecular weight standards (stained) are shown on top for each gel that was run separately. The experiment was repeated three times with the same results.

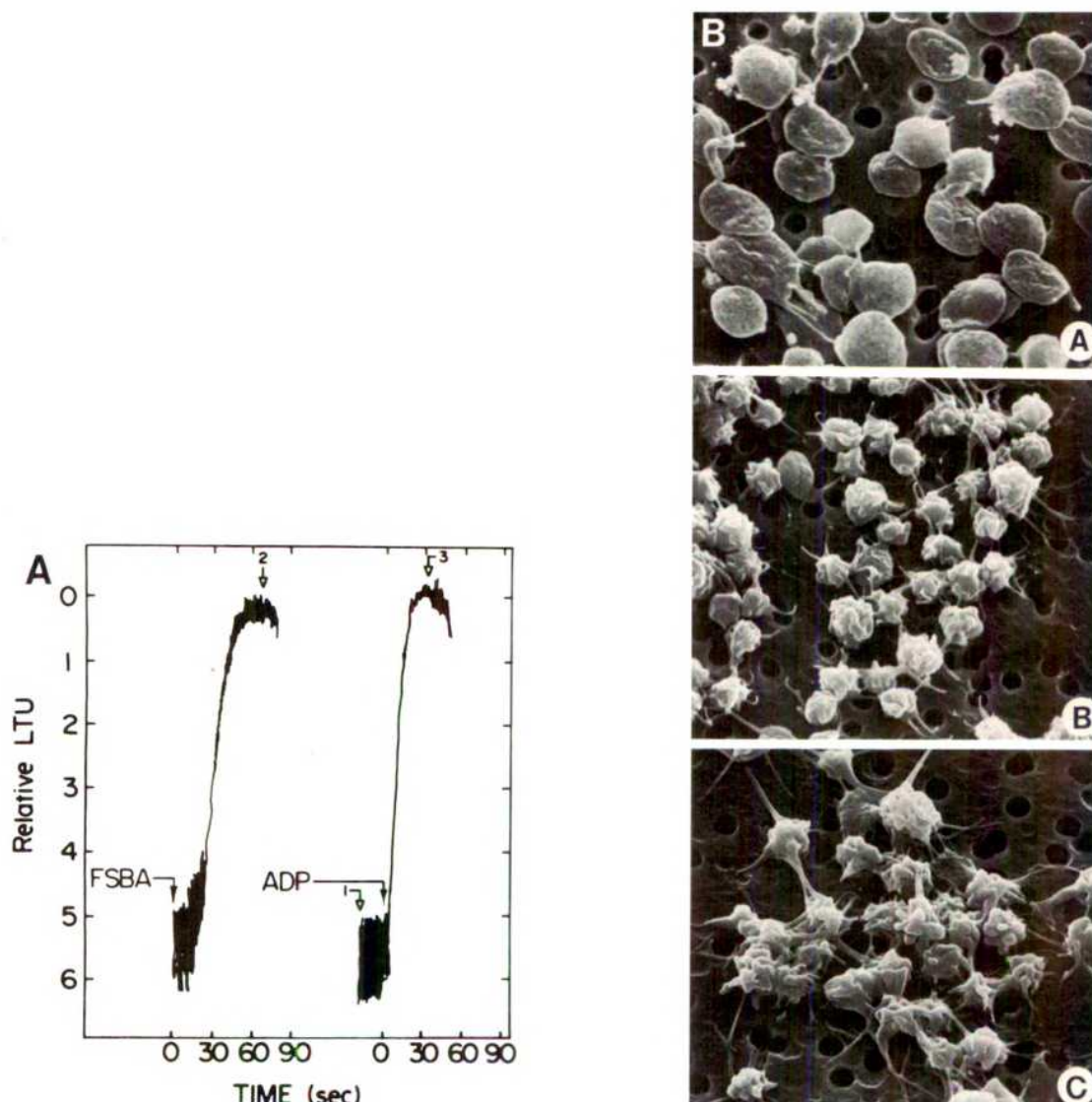


Fig 2. Comparison of FSBA and ADP-induced shape change. (A) Representative recordings of shape change selected from five similar experiments are shown. At the solid arrow (left), FSBA ($190 \mu\text{mol/L}$) is added to 1×10^8 washed platelets and shape change recorded as described in Materials and Methods section. At the solid arrow (right), ADP ($3 \mu\text{mol/L}$) is added to an aliquot of the identical platelets, and shape change recorded. (B) Scanning electron microscopy of samples taken from the experiment in frame A is a control sample that was taken before adding ADP (open arrow No. 1); frame B is a sample removed 60 seconds after FSBA at the time of maximum shape change (open arrow No. 2); frame C is a sample removed 30 seconds after ADP (open arrow No. 3) at the time of maximum shape change. Original magnification $\times 5,000$; current magnification $\times 3,250$.

cell dialysis chamber (BRL Laboratories, Model #1200MD) against phosphate buffer (0.1 mol/L , pH 7.2) containing SDS (0.1%) and EDTA (0.001 mol/L) using a $50,000 \text{ Dalton}$ (D) cutoff membrane (Spectrapor-Fisher Scientific). Dialysis was complete when the levels of radioactivity in a reference sample (identically prepared but not containing platelets) reached background levels. The samples were then removed and counted in a liquid scintillation cocktail (ACS-II, Amersham), in a Beckman Model 330 counter.

Measurement of platelet myosin phosphorylation. In order to measure light chain myosin phosphorylation, an equal volume of cold 0.6 N HClO_4 was added to the platelet sample in the aggregometer. The protein pellet was separated by centrifugation ($12,000 \times g$, three minutes) and washed once with 1 mL of H_2O . Samples were electrophoresed on alkaline-urea polyacrylamide gels as previously described.¹¹ The percentage phosphorylation was determined by

scanning the stained gel and measuring of the relative area of phosphorylated and dephosphorylated myosin light chain. The data were collected using a TRS-80 model III microcomputer and areas determined by a computer program.

RESULTS

Induction of platelet shape change by FSBA. Platelets were induced to undergo shape change by addition of ADP or FSBA. The rate and extent of shape change, measured spectrophotometrically, were very similar except that ADP induced maximum shape change at 30 seconds and FSBA ($190 \mu\text{mol/L}$) at 60 seconds (Fig 2A). Shape change was confirmed by direct examination of platelets by scanning

electromicrography (Fig 2B). Control platelets (frame A) were discoidal in shape, while ADP and FSBA-treated cells were spheroidal with numerous pseudopodia (frames B and C). The stimulation of shape change by FSBA (400 $\mu\text{mol/L}$) was not due to induction of the release reaction, since release of ATP during shape change by FSBA (190 $\mu\text{mol/L}$) was less than 0.06 $\mu\text{mol/L}$. At 400 $\mu\text{mol/L}$ the release of ATP was still less than 0.1 $\mu\text{mol/L}$. In contrast, thrombin (1 U/mL) released greater than 5 $\mu\text{mol/L}$ ATP under these conditions. Furthermore, apyrase at a concentration that completely inhibited shape change induced by ADP (0.5 $\mu\text{mol/L}$) failed to inhibit shape change induced by FSBA (400 $\mu\text{mol/L}$). The effect of FSBA was not due to stimulation of PGH_2 or thromboxane A_2 synthesis. Indomethacin (10 $\mu\text{mol/L}$), which completely inhibited shape change induced by collagen (4 $\mu\text{g/mL}$), failed to inhibit shape change induced by ADP (1 $\mu\text{mol/L}$), FSBA (400 $\mu\text{mol/L}$), or thrombin (1 U/mL).

Comparison of the relative potencies of ADP and FSBA in the induction of platelet shape change. ADP induces shape change (Fig 3) at concentrations above 0.05 $\mu\text{mol/L}$ with an $\text{EC}_{50} = 0.43 \mu\text{mol/L}$. FSBA induces platelet shape change at concentrations above 100 $\mu\text{mol/L}$ with an $\text{EC}_{50} = 220 \mu\text{mol/L}$. This difference in potencies may reflect the relative ability of the agonist to bind to the receptor due to their differing charge and structure.

Effect of FSBG on platelet shape change. To ascertain whether the effect of FSBA was specific and in particular the role of the sulfonyl fluoride group, a structural analog of FSBA, FSBG (differing only in the nature of purine base) was tested for the ability to induce platelet shape change. FSBG has no measurable effect on platelets even at concentrations of 500 $\mu\text{mol/L}$ when dissolved in similar final concentrations (<2.0%) of DMF.

Evidence for specific interaction of FSBA with platelet ADP receptor-mediating shape change. In order to further determine whether the induction of platelet shape change by

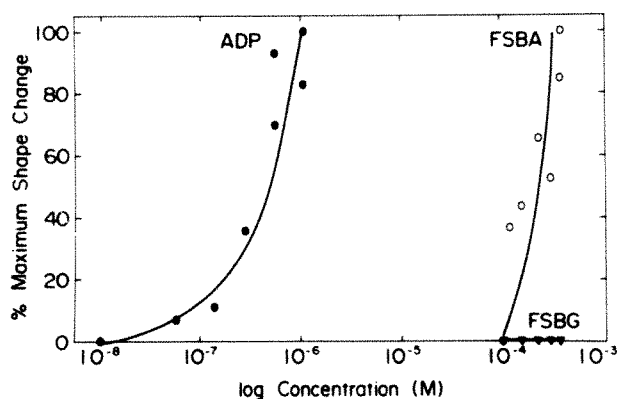


Fig 3. Effect of various concentrations of ADP, FSBA, and FSBG on induced platelet shape change. Each compound was tested at a wide range of concentrations for ability to produce shape change as in Fig 2A. For comparison, each is normalized to the percent of maximum shape change and plotted as a log of concentration. The data are from a single experiment and are representative of three such experiments.

FSBA is due to specific interaction of nucleotide affinity analog with the platelet surface, ADP receptor inhibition studies using ATP were carried out. ATP has been shown to be a specific antagonist of ADP-mediated platelet shape change and aggregation.¹² Platelets were washed as described above, and FSBA-mediated shape change was carried out at various concentrations of FSBA in the presence and absence of ATP (600 $\mu\text{mol/L}$), a concentration which inhibited 50% shape change induced by ADP (3 $\mu\text{mol/L}$) and 100% of that induced by 1 $\mu\text{mol/L}$ ADP. As shown in Table 1, ATP proved capable of inhibiting FSBA-mediated shape change to as little as 22% of maximum. Note that at the highest concentration of FSBA, the inhibition by ATP was decreased similar to the competitive mechanism documented for ATP inhibition of ADP aggregation.¹²

Effects of low concentrations of FSBA on platelets. The ability of FSBA to induce platelet shape change at concentrations above 100 $\mu\text{mol/L}$ occurs within one minute after the addition of the compound to stirred platelet suspensions. In contrast, FSBA (40 $\mu\text{mol/L}$) does not produce shape change even after 60 minutes of incubation. However, FSBA (40 $\mu\text{mol/L}$) can inhibit ADP-mediated shape change progressively over time with 50% inhibition at 10 minutes and total inhibition of shape change occurring at 20 minutes (Fig 4A). Using different platelet donors in ten additional experiments, the time necessary for total inhibition varied between 15 and 40 minutes. Platelets incubated with FSBA under these conditions failed to show shape change, as assessed by scanning electron microscopy,² after stimulation with ADP (10 $\mu\text{mol/L}$). Platelets stimulated with ADP (10 $\mu\text{mol/L}$) showed shape change after 60 minutes of incubation with FSBG or DMF equal to that without incubation. Figure 4B displays a time course of covalent incorporation of the compound into platelet membranes. The maximum incorporation was 60.4 pmol SBA/ 10^8 platelets. Comparing Figs 4A and 4B, it is evident that 36 pmol SBA incorporated per 10^8 platelets is sufficient for maximum inhibition.

Correlation of induction of shape change with incorporation of [^3H]-SBA at a high concentration of FSBA. When cells were treated with high concentrations of FSBA (400 $\mu\text{mol/L}$), they changed shape very rapidly (Fig 5), as demonstrated by superposition of the time course of the incorporation of [^3H]-SBA into platelet membrane protein as compared with the actual spectrophotometric recording of

Table 1. Inhibition of FSBA-induced Shape Change by ATP

FSBA ($\mu\text{mol/L}$)	Percent Maximum Rate of Change	
	- ATP	+ ATP
100	44	22
200	85	26
300	100	35
400	85	74

Platelets were washed as described in Materials and Methods section. ATP (600 $\mu\text{mol/L}$) was added, followed immediately by FSBA at various concentrations, and the rate of change was measured and normalized to percent of maximum observed in the presence of the corresponding concentration of DMF. This experiment is representative of three such experiments.

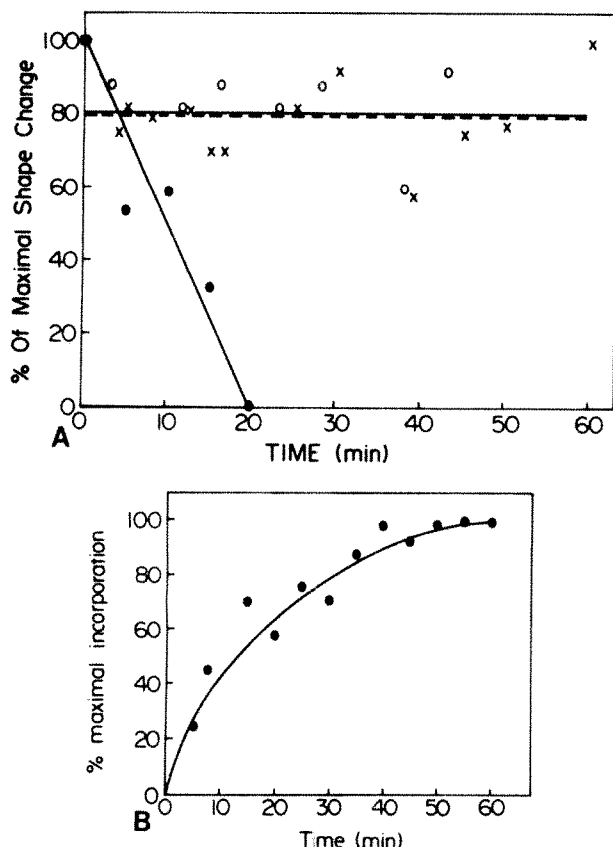


Fig 4. Inhibition by FSBA on platelet shape change and correlation with covalent incorporation. Platelets were incubated with FSBA (40 $\mu\text{mol/L}$) (filled circles), FSBG (40 $\mu\text{mol/L}$) (open circles), or DMF (x). The 100% point was determined in the presence and absence of DMF and was identical. (A) At various time intervals, aliquots of the reaction mixture were withdrawn and the cells were tested for their ability to undergo shape change in response to ADP (10 $\mu\text{mol/L}$). (B) An additional aliquot of samples was simultaneously incubated with $[^3\text{H}]\text{-FSBA}$ (40 $\mu\text{mol/L}$) to determine the extent of covalent incorporation of $[^3\text{H}]\text{-SBA}$ in the membrane receptor protein (see Materials and Methods).

platelet shape change. The results demonstrate that only 2 pmol/ 10^8 platelets of $[^3\text{H}]\text{-SBA}$ was incorporated into membrane polypeptide at the onset of platelet shape change and 4 pmol/ 10^8 cells at maximum shape change. From the data in Figures 4A and 4B, this concentration is not high enough to produce detectable inhibition of ADP-induced shape change.

Effect of the covalent incorporation of SBA on shape change induced by FSBA. Two possibilities exist to explain the previous observation that at the time when shape change is induced by 400 $\mu\text{mol/L}$ FSBA, insufficient SBA was incorporated to block ADP-induced shape change. Either additional FSBA is noncovalently bound to the same site to induce shape change or FSBA is noncovalently bound to a second site distant from aggregin. To distinguish these alternatives, we incubated washed platelets with FSBA (400 $\mu\text{mol/L}$) for 40 minutes to insure extensive covalent incorporation, and in parallel the same platelets were incubated with the solvent DMF. FSBA (50 to 400 $\mu\text{mol/L}$) was then added to the platelets, and the maximum extent of shape change

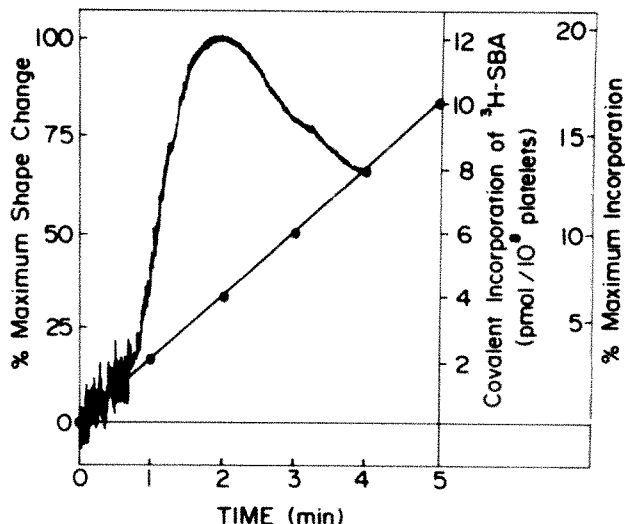


Fig 5. Correlation of incorporation of $[^3\text{H}]\text{-SBA}$ into platelets with induction of shape change by high concentration of FSBA. $[^3\text{H}]\text{-FSBA}$ (400 $\mu\text{mol/L}$) was incubated with washed platelets at various times. The actual tracing (continuous line, left axis) is presented. Aliquots were withdrawn and assayed for covalent incorporation of $[^3\text{H}]\text{-SBA}$ (filled circles) and expressed on the right axes as both absolute incorporation as well as percent maximum incorporation (as measured in Fig 4B).

(Fig 6) as well as the maximum rate of shape change (data not shown) were measured. The platelets incubated with DMF underwent shape change, the maximum extent of which was proportional to the log of the FSBA concentration ($r = 0.88$, $P < .01$). Similar findings were observed for the maximum rate of shape change ($r = 0.88$, $P < .01$). In contrast, over the same range of FSBA concentration shape change was completely inhibited, suggesting that the covalent and noncovalent sites for FSBA were similar.

The effect of FSBA on phosphorylation of platelet myosin. Phosphorylation of myosin light chain is an early response of platelets to stimulation by an agonist and has been shown to correlate with ADP-induced platelet shape change.¹³ We have investigated whether the high concentrations of FSBA that induce platelet shape change also induce myosin phosphorylation and whether the low doses of FSBA that do not induce shape change also fail to induce myosin phosphorylation. When platelets were stimulated with 400 $\mu\text{mol/L}$ FSBA, shape change occurred, and as in previous experiments, the response was maximal at about 90 seconds. The measurement of myosin phosphorylation (Fig 7A) closely paralleled the shape change response. The phosphorylation was qualitatively similar to that seen with ADP in that after shape change was complete, the level of phosphorylation gradually returned to basal levels (Fig 7B). At 90 seconds about 3 to 4 pmol of FSBA per 10^8 cells would be incorporated into the platelet membrane (Fig 5). At 40 $\mu\text{mol/L}$, the same incorporation requires five to ten minutes. When platelets were incubated with 40 $\mu\text{mol/L}$, no shape change or myosin phosphorylation was observed (Fig 7A, open circles). In fact, no phosphorylation was found at times up to 60 minutes when FSBA incorporation was maximal.

The ability of FSBA to block ADP-induced myosin phos-

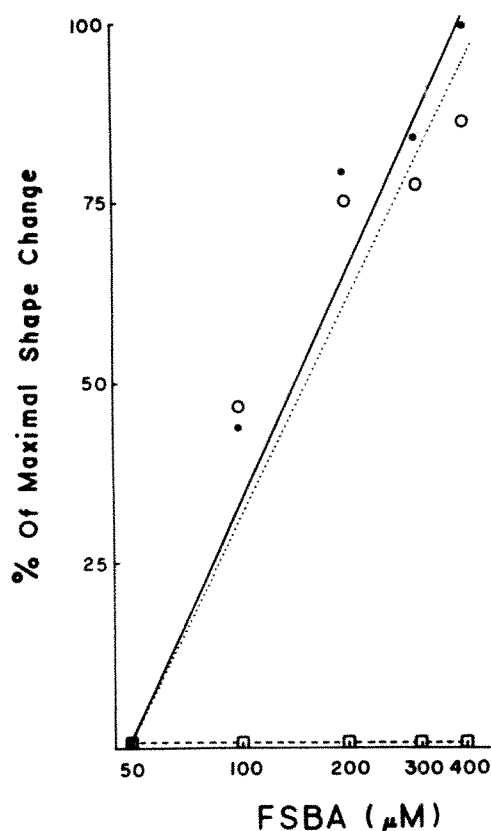


Fig 6. Effect of FSBA and FSBG under conditions of covalent incorporation on shape change induced by FSBA. Washed platelets (5×10^8 /mL) incubated with FSBA (400 μ mol/L) (○) or FSBG (○) (100 μ mol/L) or DMF (●) for 60 minutes. The platelets were then diluted to 1×10^8 with the same suspending buffer, and FSBA (50 to 400 μ mol/L) was added with stirring and shape change measured as in the Materials and Methods section. Each point is the mean of three separate experiments.

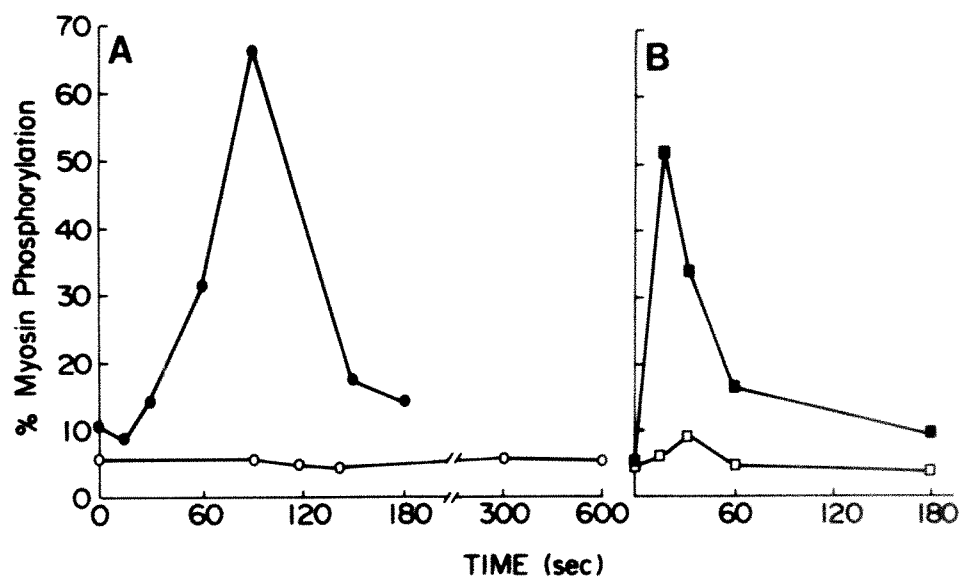
phorylation was also tested (Fig 7B). ADP (10 μ mol/L) produced rapid shape change that, as previously shown, was accompanied by a parallel increase in myosin phosphorylation.¹³ Platelets incubated with 40 μ mol/L FSBA for one hour no longer exhibited a shape change when challenged with ADP (open circles). The ADP-induced change in myosin phosphorylation was inhibited by over 90% in the FSBA-treated cells.

DISCUSSION

The identification of the ADP receptor responsible for the induction of ADP-mediated shape change and aggregation has been studied using a number of approaches. Any measurement of direct binding of ADP to platelet membranes is complicated, since intact platelets metabolize ADP to adenosine, which is transported into the platelets or ATP, which interferes with binding. Born and Feinberg¹⁴ measured 88,000 "high affinity sites" for ^{14} C-ADP on platelets in plasma; however, they did not differentiate between bound ^{14} C-ADP and ^{14} C-adenosine formed from the labeled ADP and taken up by the platelet. Lipps¹⁵ measured the direct binding of radiolabeled ADP to intact platelets and demonstrated that the binding constant of the higher affinity site was in the range of ADP concentration necessary to promote platelet shape change and aggregation. However, since a significant amount of ADP added to the cells was converted to ATP, a known competitive antagonist of ADP, these experiments must be interpreted with caution.

Binding of ^{14} C-ADP to 100,000 sites on isolated plasma membranes was recorded by Nachman and Ferris.¹⁶ However, the sites identified would have included the binding of ADP to nucleotide binding proteins on the inside of the isolated membranes, since Bennett et al.⁵ using the adenosine nucleotide affinity analog FSBA, demonstrated the existence

Fig 7. Effect of FSBA on platelet myosin light chain phosphorylation. (A) FSBA was incubated with washed platelets with stirring at 400 μ mol/L (●) and 40 μ mol/L (○). At various times, aliquots were withdrawn for determination of myosin light chain phosphorylation. (B) Platelets were preincubated with DMF (■) or FSBA, 40 μ mol/L (□) for 40 minutes at 37°C. At time 0, ADP (5 μ mol/L) was added. At various times, aliquots were withdrawn for determination of myosin light chain phosphorylation. Platelets preincubated without addition of DMF or FSBA behaved similarly to those incubated with DMF (data not shown). These data are representative of three similar experiments.



of at least four nucleotide binding proteins (including actin and myosin) in isolated platelet membranes.⁴ An ADP binding protein ($M_r = 60$ kDa) has been isolated by Adler and Handin¹⁷ from platelet membranes by freezing and thawing, but no further studies relating this protein to an ADP receptor in intact platelets were performed.

In an attempt to more clearly define the ADP receptor on the platelet cell surface responsible for activation, Bennett et al⁵ used the adenosine nucleotide affinity analog FSBA, which does not penetrate the cell membrane. FSBA (100 $\mu\text{mol/L}$) inhibited ADP-mediated platelet shape change, and in intact platelets, only aggregin was covalently modified. This study confirms these findings and shows that even at 400 $\mu\text{mol/L}$ FSBA the same polypeptide is modified (Fig 1). Subsequently, Figures et al⁶ demonstrated that FSBA (100 μM) was an inhibitor of ADP-mediated aggregation as well as ADP-induced exposure of latent platelet fibrinogen receptors.

The present study further delineates the specificity and consequences of the FSBA-platelet interaction. We have confirmed, both by spectrophotometric assay and scanning electron microscopy (Fig 2), the ability of FSBA (400 $\mu\text{mol/L}$) to induce platelet shape change and further demonstrated that it induces myosin phosphorylation (Fig 7). FSBA (200 to 400 $\mu\text{mol/L}$) does function as an agonist similar to ADP, but at a higher concentration (Figs 3, 7). The specificity of the interaction of FSBA and platelets is demonstrated by the failure of FSBG to produce shape change (Fig 3) as well as by the inhibition of FSBA-mediated shape change by ATP, a known competitive inhibitor of ADP responses of platelets (Table 1). FSBA appears to function as a partial agonist, and the protein interacting with FSBA may fulfill the criteria of a receptor with regard to the specificity of binding as well as the correlation of cellular responses with the concentration of FSBA. However, FSBA (40 $\mu\text{mol/L}$) is also capable of acting as an antagonist of ADP-mediated responses^{2,5,6} when incubated for prolonged periods of time. The antagonistic effect of FSBA correlates well with the covalent incorporation of the molecule into the platelet membrane protein (Fig 4), with total inhibition occurring at 20 to 40 minutes of incubation with FSBA (40 $\mu\text{mol/L}$). Bennett et al,⁵ using platelets suspended in plasma,

observed inhibition of ADP-mediated shape change with FSBA at periods as short as ten seconds. However, such inhibition could be prevented by using washed platelets in the presence of adenosine deaminase,² indicating that hydrolytic breakdown of FSBA to adenosine and fluorosulfonylbenzoic acid may be enhanced by plasma esterases. We have therefore carried out all of our incubations in the presence of adenosine deaminase to obviate this effect. As with the agonist effect of FSBA, we have demonstrated that the antagonism of the ADP response by FSBA is specific by comparison with the lack of an effect with FSBG.²

The ability of FSBA to act as both an agonist and antagonist in platelets raises the question of its mechanism. As shown in Fig 5, in which 400 $\mu\text{mol/L}$ FSBA is used to induce shape change, the amount of covalent incorporation at two minutes is less (6% of maximum) than that achieved at longer times with 40 $\mu\text{mol/L}$ FSBA (60% of maximum, Fig 4). This result implies that the covalent reaction probably does not induce the platelet activation. The initial noncovalent interaction between FSBA and platelets before covalent incorporation is probably the trigger for shape change response, which only occurs at the high FSBA concentration. That this is probably at the same site is shown by the ability of FSBA (400 $\mu\text{mol/L}$, 60 minutes) to block FSBA (100 to 400 $\mu\text{mol/L}$)-induced shape change (Fig 6). Specificity is confirmed by the failure of FSBG under the same conditions (400 $\mu\text{mol/L}$, 60 minutes) to inhibit FSBA-induced shape change. FSBA (100 $\mu\text{mol/L}$, 60 minutes) can also completely inhibit shape change produced by a second addition of FSBA (100 $\mu\text{mol/L}$) and partially (60%) inhibit 200 to 400 $\mu\text{mol/L}$ FSBA (mean of three experiments; data not shown).

The inability of lower concentrations of FSBA to induce myosin phosphorylation appears to be related to the rates of phosphorylation and dephosphorylation. Shape change could therefore be observed only at concentrations of FSBA high enough (>100 $\mu\text{mol/L}$) to activate myosin light chain kinase rapidly enough so that the rate of phosphorylation could exceed the rate of dephosphorylation. However, low concentrations of FSBA (40 to 100 $\mu\text{mol/L}$) that covalently modify the receptor prevent the binding of ADP, which then fails to induce either shape change or myosin phosphorylation.

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Ca²⁺ Permeability and Cytosolic Ca²⁺ Concentration Are Not Impaired in β -Thalassemic and Hemoglobin C Erythrocytes

By M.D. Rhoda, F. Galacteros, Y. Beuzard, and F. Giraud

Total calcium content, determined by atomic absorption spectroscopy, Ca²⁺ influx, and cytosolic free Ca²⁺ concentration ([Ca]_i), estimated by a method involving the incorporation of a Ca²⁺ chelator (Quin 2), were measured in erythrocytes from β -thalassemic (β -thal) and hemoglobin C (CC) patients. Elevation of the total calcium content was observed in the cells from all patients, particularly in CC and splenectomized β -thal. However, [Ca]_i was within the normal range (~25 nmol/L) in all the pathologic cells. Ca²⁺ influx in CC cells and in cells from nonsplenectomized β -thal patients was also within the same range as that observed in control erythrocytes. In cells from splenectomized β -thal patients, the kinetic of ⁴⁵Ca influx was biphasic, indicating the existence of two pools of exchangeable Ca²⁺. Density fractionation of the cells from one splenec-

tomized β -thal patient showed that the rapid pool corresponded to the lightest cell fraction, which was also found to have the highest calcium content. The dense cells exhibited a normal Ca²⁺ influx as well as a smaller increase in total calcium content. It is suggested that, as in sickle cell anemia, the excess of Ca²⁺ in β -thal cells is not free in the cytoplasm but trapped within endocytic vacuoles, especially in a population of abnormal cells that are normally removed by the spleen. In CC patients, who have a functional spleen, a different mechanism could be responsible for the calcium retention. In conclusion, the present results demonstrate that in these two cases of hemolytic anemia associated with high calcium content, Ca²⁺ permeability and the level of cytosolic Ca²⁺ are normal.

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IN SOME hemolytic anemias, the total calcium concentration in erythrocytes greatly exceeds that in normal healthy cells. In sickle cell anemia, this high calcium content is associated with an increase in Ca²⁺ fluxes¹⁻³ but not with an increase in cytosolic free Ca²⁺ concentration ([Ca]_i).^{3,4} This is because the excess of Ca²⁺ was shown to be concentrated in endocytic inside-out vesicles.^{5,6} In β -thalassemia (β -thal) and hemoglobin C (CC) disease, red cells have also been reported to have a high calcium content^{7,8} and alterations in ionic permeabilities.⁹⁻¹² To obtain more information about the state of Ca²⁺ in these pathologic cells, we measured both the Ca²⁺ influx and the [Ca]_i by a method using the intracellular incorporation of the Ca²⁺ chelator, Quin 2. The latter leads to increasing the exchangeable Ca²⁺ pool without altering the membrane properties or the metabolic state of the cells.¹³⁻¹⁶ When applied under steady-state conditions of internal Ca²⁺, the method permits the determination of the characteristics of the exchangeable Ca²⁺ pool(s) (size and rate constant) from the kinetic analysis of the ⁴⁵Ca tracer concentration.^{3,16}

The present results indicate that Ca²⁺ influx and cytosolic Ca²⁺ concentration are not altered in CC and β -thal cells in the absence of splenectomy but that splenectomy in β -thal unmasks a population of cells with abnormal Ca²⁺ permeability.

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MATERIALS AND METHODS

Patients and Controls

Blood samples from five patients homozygous for hemoglobin C and six patients with β -thalassemia intermedia were obtained with informed consent. Two β -thalassemic patients were not splenectomized and four were splenectomized. Splenectomy was performed at least 1 year before the present study. Control erythrocytes (hemoglobin A) were collected from 16 healthy volunteers in our laboratory. Blood from two splenectomized adults and three reticulocyte-rich control subjects, free of red cell disorder, was also used. All blood samples were collected into heparinized tubes.

Measurement of Total Calcium Content

Total calcium content was determined by atomic absorption spectroscopy on freshly drawn erythrocytes washed four times with 10 vol of ice-cold Ca²⁺ free isotonic saline. The cell suspension (hematocrit 90%) was precipitated with 4 vol of 10% (wt/vol) trichloroacetic acid containing 10,000 ppm lanthanum.

Chelator Loading and Ca²⁺ Flux Measurements

Preparation of erythrocytes. Freshly drawn erythrocytes were washed three times in the following solution: 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 10 mmol/L glucose, 10 mmol/L HEPES (pH 7.4) (buffer A). These were kept overnight at 4°C in the same medium at a hematocrit of ~20%. Before chelator loading, erythrocytes were incubated in an ATP regenerating system (2 mmol/L adenine, 10 mmol/L inosine, in buffer A, 45 minutes at 37°C, hematocrit 15%) and then washed three times in buffer A. Aliquots were taken for ATP measurement (UV test-combination, Boehringer-Mannheim, West Germany).

Chelator loading. Quin-2-AM (Lancaster Synthesis, Morecambe, UK) incorporation was as described by Lew et al¹⁴ except that 1 mmol/L CaCl₂ was present in the incubation buffer. Cells were incubated (45 minutes at 37°C, hematocrit 30%) with Quin-2-AM (300 μ mol/L cells) and then washed three times in buffer A. Aliquots were taken for measurement of ATP and total intracellular chelator concentration ([Chel]_i). This latter was determined from its fluorescence signal observed after its extraction from the cells and removal of hemoglobin (as described in ref. 18). The concentration of Quin-2 was obtained by comparison with a standard curve obtained under the same conditions from unknown concentrations of Quin-2 (Lancaster Synthesis, Morecambe, UK).

Ca²⁺ influx. Chelator-loaded cells washed three times in buffer A were resuspended in the same buffer containing ⁴⁵Ca (CEA-ORIS, France) (hematocrit 10%). At different times duplicate aliquots of the cell suspensions (100 to 200 μL) were treated as described in ref 3 (three washes in 10 mL buffer A, addition of trichloroacetic acid to the cell pellet and ⁴⁵Ca counting of the supernatant by liquid scintillation).

Validation of the Chelator Method and Calculations

The method used to determine Ca²⁺ influx and [Ca]_i involves the incorporation of a calcium chelator (Quin-2) that creates a measurable exchangeable Ca²⁺ pool. A low concentration of external chelator (300 μmol/L cells) was chosen in order to prevent massive ATP depletion¹⁷ but it was sufficient to measure [Ca]_i properly (see below). The lack of effect of this procedure on Ca²⁺ influx and on [Ca]_i has previously been demonstrated.¹³⁻¹⁶

Ca²⁺ influx. In our experimental conditions, the Ca²⁺ exchange system was in steady-state, since all the steps, including chelator loading, were carried out in the presence of 1 mmol/L CaCl₂, and thus ⁴⁵Ca movements can be analyzed, assuming that cell Ca²⁺ constituted a one-open compartment system.

Total exchangeable Ca²⁺ (⁴⁵Ca_{eq}, μmol/L cells) was calculated from the cellular ⁴⁵Ca radioactivity at isotopic equilibrium (cpm/L cells) divided by the specific activity of ⁴⁵Ca in the external medium (cpm/μmol). The values of ⁴⁵Ca uptake at each time, divided by the

specific activity of ⁴⁵Ca in the medium, will be referred to as ⁴⁵Ca_i. By plotting log (⁴⁵Ca_{eq} - ⁴⁵Ca_i) as a function of time, one can determine *k* (h⁻¹), the apparent rate constant for Ca²⁺ influx (see Fig 1). Ca²⁺ influx (μmol/L cells/h) was calculated from *k* · ⁴⁵Ca_{eq}. In some cases, these plots were biphasic. Extrapolation of the linear part of the curve at zero time permitted the calculation of the size and the rate constant (*k*₂, h⁻¹) of the slowly exchangeable Ca²⁺ pool. Curve-peeling method was used to calculate these parameters for the rapidly exchangeable compartment (rate constant *k*₁, h⁻¹).

[Ca]_i/[Ca]_e was calculated from the equilibrium reaction:



in which [Chel]_i is the intracellular concentration of free chelator and *K_D* the dissociation constant of the Ca - chelator complex ([Ca - Chel]).

$$K_D = \frac{[Ca]_i \times [Chel]_i}{[Ca - Chel]} \text{ or } [Ca]_i = K_D \frac{[Ca - Chel]}{[Chel]_i} \quad (1)$$

Total exchangeable Ca²⁺ (⁴⁵Ca_{eq}) and total intracellular chelator ([Chel]_i) are, respectively equal to:

$$^{45}\text{Ca}_{eq} = [Ca]_i + [Ca - Chel] \quad (2)$$

$$[Chel]_i = [Chel]_f + [Ca - Chel] \quad (3)$$

In chelator-loaded erythrocytes, virtually all the exchangeable

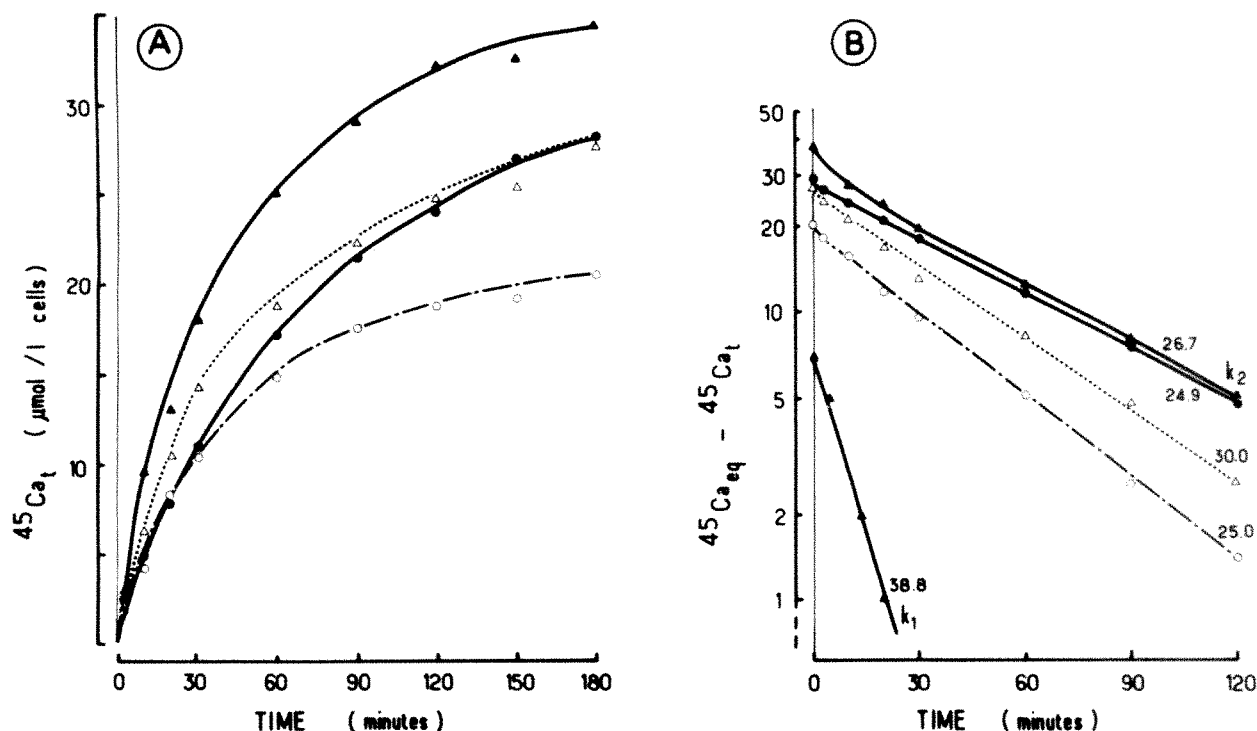


Fig 1. ⁴⁵Ca uptake by Quin-2-loaded AA (●), CC (○), and β-thal cells from splenectomized (▲) or nonsplenectomized patient (△). Erythrocytes were loaded with Quin-2-AM in the presence of 1 mmol/L CaCl₂, washed, and incubated with 1 mmol/L ⁴⁵CaCl₂. ⁴⁵Ca uptake was measured at different times (⁴⁵Ca_t) up to isotopic equilibrium (⁴⁵Ca_{eq}). Equilibrium was usually reached between 180 and 210 minutes, depending both on the amount of chelator incorporated and on the rate of Ca²⁺ influx. ⁴⁵Ca_i and ⁴⁵Ca_{eq} were expressed as μmol/L cells by dividing the cell radioactivity (cpm/L cells) by the specific radioactivity of ⁴⁵Ca in the external medium (cpm/μmol) (A). Kinetics were analyzed assuming a one-open compartment system according to the equation: ⁴⁵Ca_t = ⁴⁵Ca_{eq} (1 - e^{-kt}) or Log(⁴⁵Ca_{eq} - ⁴⁵Ca_t) = -*k*₁ + Log⁴⁵Ca_{eq}. The value of the rate constant, *k*, was obtained from the plot of Log(⁴⁵Ca_{eq} - ⁴⁵Ca_t) v time (B). Since ⁴⁵Ca_{eq} depends on the amount of incorporated chelator, *k* is an apparent rate constant, and Ca²⁺ influx can only be calculated from *k* · ⁴⁵Ca_{eq} in each batch of cells. In β-thal from the splenectomized patient, two slopes were observed (*k*₁ and *k*₂). The values of Ca²⁺ influx (μmol/L cells/h) are indicated on the graph.

Ca^{2+} will be bound to the chelator, all other possible Ca^{2+} ligands (ATP or 2,3-DPG) having a lower binding capacity (see ref. 14). If $[\text{Chel}]_i$ is large enough to create a total exchangeable Ca^{2+} pool ($^{45}\text{Ca}_{\text{eq}}$) at least ten-fold greater than the preexisting exchangeable Ca^{2+} pool ($1 \mu\text{mol/L}$ in AA cells and about $3 \mu\text{mol/L}$ in pathological cells, results not shown), $^{45}\text{Ca}_{\text{eq}}$ can be considered to be equal to chelator-bound Ca^{2+} . Under our experimental conditions (concentration of external chelator $300 \mu\text{mol/L}$ cells, yield of chelator incorporation 50% to 70%) $^{45}\text{Ca}_{\text{eq}}$ was about $30 \mu\text{mol/L}$ cells (Fig 1). Then equations (2) and (3) become:

$$^{45}\text{Ca}_{\text{eq}} = [\text{Ca} - \text{Chel}] \quad (2')$$

$$[\text{Chel}]_i = [\text{Chel}]_t - ^{45}\text{Ca}_{\text{eq}} \quad (3')$$

and equation (1):

$$[\text{Ca}]_i = K_D \frac{^{45}\text{Ca}_{\text{eq}}}{[\text{Chel}]_t - ^{45}\text{Ca}_{\text{eq}}}$$

$[\text{Chel}]_i$ and $^{45}\text{Ca}_{\text{eq}}$ were determined as described above. K_D was taken as 115 nmol/L , a value determined under conditions mimicking cell cytoplasm,¹⁵ which well agreed with that found in erythrocytes in our previous work.³

Other methods. A discontinuous gradient of Stractan (density range 1.060 to 1.130) was used to fractionate the cells from splenectomized β -thal patients as described by Corash et al.¹⁹ Two fractions were isolated: F_1 $1.060 < d < 1.070$ and F_2 $d > 1.074$.

ATP was measured, after perchloroacetic acid extraction, with a UV test combination (Boehringer Mannheim Diagnostica, RFA). Cell volume was estimated from the hematocrit or from the hemoglobin content.

RESULTS

Total Calcium Content

In control (AA) erythrocytes, total calcium content was $7.3 \pm 1.1 \mu\text{mol/L}$ cells (Table 1). Large increases (approximately eightfold) were observed in cells from hemoglobin C (CC) and from splenectomized β -thalassemic (β -thal) patients. A moderate increase was observed in β -thal cells from nonsplenectomized patients. Splenectomy, per se, did not induce any change in the calcium content of AA cells. The calcium content was also measured in density-fractionated cells from one splenectomized β -thal patient (F_1 light cells, F_2 dense cells, see Materials and Methods). The calcium content in F_1 , which contained the most abnormal cells, was higher than that in F_2 fraction.

ATP Content

ATP was measured before and after chelator loading and at the end of the Ca^{2+} influx experiments. The chelator

incorporation reduced the level of ATP by 40% in AA cells but only by 20% to 30% in CC and β -thal cells (results not shown). This level was partially restored in AA cells and almost totally in pathologic cells at the end of the Ca^{2+} influx experiment. The initial level of ATP was 0.53 ± 0.05 , 0.58 ± 0.06 , and $0.46 \pm 0.06 \text{ mmol/L}$ cells, respectively, in AA, CC, and β -thal cells.

Ca^{2+} Influx

AA, β -thal, and CC cells were loaded with Quin-2-AM, washed, and incubated with ^{45}Ca . The kinetics of ^{45}Ca uptake are shown in Fig 1A. In all types of cells, the ^{45}Ca content ($^{45}\text{Ca}_i$ expressed in $\mu\text{mol/L}$ cells) reached an equilibrium value ($^{45}\text{Ca}_{\text{eq}}$) after about 180 minutes. The semilogarithmic plot of $^{45}\text{Ca}_{\text{eq}} - ^{45}\text{Ca}_i = f(t)$ (Fig 1B) was linear in AA, CC, and β -thal cells (from nonsplenectomized patients), indicating that exchangeable Ca^{2+} in these cells behaved as one homogenous pool. This plot was biphasic in β -thal cells from splenectomized patients, revealing the existence of two exchangeable Ca^{2+} pools with rate constants $k_2 = 0.89 \text{ h}^{-1}$ and $k_1 = 5.55 \text{ h}^{-1}$ in the experiment of Fig 1. As shown in Table 2, Ca^{2+} influx was 26 to $31 \mu\text{mol/L}$ cells/h (28.2 ± 1.7 , $n = 16$) in AA cells. It remained within the same range in the erythrocytes from CC and nonsplenectomized β -thal patients. In the cells from the splenectomized β -thal patient, the influx corresponding to k_2 , which concerned about 80% of the total exchangeable Ca^{2+} , was equal to that found in AA cells (Fig 1B; Table 2). The influx corresponding to k_1 , which concerned only 20% of the pool, was more difficult to estimate because of imprecision in the determination of k_1 . This Ca^{2+} influx was within the range of 35 to $70 \mu\text{mol/L}$ cells/h (Table 2).

Experiments were then conducted in order to investigate whether the kinetic heterogeneity of the ^{45}Ca exchange in β -thal cells from splenectomized patients was due to cellular heterogeneity. Since the splenectomized β -thal patients had a high reticulocyte count, Ca^{2+} influx was measured in AA cells containing a similar increase in reticulocytes (about 5%). The kinetic was monophasic, and no significant change in the ^{45}Ca influx was observed (Table 2). Similar experiments were done with cells from splenectomized control, and again no difference was observed (Table 2). Finally, Ca^{2+} influx was measured in density fractionated cells from one splenectomized β -thal patient. In the F_1 fraction, corresponding to the most abnormal cells, the biexponential kinetic was maintained. However, a monoexponential kinetic was observed in the F_2 fraction with a nearly normal value for

Table 1. Total Calcium Content ($\mu\text{mol/L}$ Cells)

AA	AA (sp)	CC	β -Thal (non sp)	β -Thal (sp)		
				Total	F_1^*	F_2^*
7.3 ± 1.1	$4.4 - 8.8$	59.5 ± 4.0	$21.5 - 14.4$	50.2 ± 15.3		
(6)	(2)	(4)	(2)	(3)		
				39 (1)	46 (1)	36 (1)

Values are mean $\pm$ SE of n experiments (number in parentheses) or individual values when $n = 1$ or 2, in nonsplenectomized and splenectomized controls and β -thal subjects and in CC patients.

F_1^* and F_2^* correspond to two subpopulations of β -thal cells isolated by density gradient from one splenectomized patient. F_1 corresponds to the lightest and most abnormal cells (see text).

Abbreviations: sp, splenectomized; non sp, nonsplenectomized.

Table 2. Ca^{2+} Influx and Cytosolic Free Ca^{2+} Concentration $[\text{Ca}]_i$

	Ca^{2+} Influx ($\mu\text{mol/L cells/h}$)		$[\text{Ca}]_i$ (nmol/L cells)	
AA	26.2 ± 2.8	(5)	25.9 ± 2.5	(3)
CC	24.1 ± 1.6	(5)	20.2 ± 2.1	(3)
AA	26.8 ± 3.1	(5)	24.3 ± 2.1	(5)
β -thal (non sp)	$29.1 - 27.7$	(2)	$28.1 - 31.3$	(2)
β -thal (sp)	$27.9 \pm 4.3^*$	(4)	$21.5 \pm 0.8^\dagger$	(3)
	$45.2 \pm 9.6^\ddagger$	(4)		
AA	31.5 ± 2.5	(6)	26.0 ± 1.2	(6)
AA (sp)	31.5 ± 4.0	(3)	22.3 ± 1.2	(3)
AA (retic)	34.9 ± 3.9	(3)	$24.2 - 22.7$	(2)

Ca^{2+} influx and $[\text{Ca}]_i$ were determined in Quin-2-loaded cells incubated with 1 mmol/L ^{45}Ca . Chelator incorporation created an internal pool of exchangeable Ca^{2+} ($^{45}\text{Ca}_{\text{ex}}$) easily measurable with the radiotracer. Ca^{2+} influx was calculated from the kinetic analysis of ^{45}Ca uptake (see Fig 1). Because of the low endogenous Ca^{2+} -binding capacity of erythrocytes at low $[\text{Ca}]_i$, virtually all exchangeable ^{45}Ca will be bound to Quin-2, and $[\text{Ca}]_i$ can be calculated from $[\text{Ca}]_i = K_D \cdot ^{45}\text{Ca}_{\text{ex}}/([\text{Chel}]_i - ^{45}\text{Ca}_{\text{ex}})$, with K_D the dissociation constant of the Ca-Quin-2 complex (115 nmol/L) (see Materials and Methods). Values are means $\pm$ SE of n experiments (number indicated in parentheses) or individual values when $n = 2$.

Abbreviation: AA (retic), AA cells containing 53% of reticulocytes.

* Ca^{2+} influx corresponding to k_2 (see Fig 1 and text).

$^\dagger\text{Ca}^{2+}$ influx corresponding to k_1 (see Fig 1 and text).

$^\ddagger[\text{Ca}]_i$ was calculated from $^{45}\text{Ca}_{\text{ex}}$ obtained by extrapolation of k_2 .

Ca^{2+} influx (results not shown). The results indicated that the kinetic heterogeneity could be attributed to the presence of a subpopulation of cells contained in the F_1 fraction and confirmed that Ca^{2+} influx into the majority of the cells in splenectomized β -thal patients (F_2 fraction) was normal.

$[\text{Ca}]_i$

$[\text{Ca}]_i$ was calculated as indicated in Methods. No significant differences in $[\text{Ca}]_i$ were observed between normal AA cells and the pathologic CC and β -thal cells (Table 2), nor was $[\text{Ca}]_i$ changed in AA cells from splenectomized control or reticulocyte-rich patients.

DISCUSSION

In agreement with previous observations,^{7,8} the total calcium content in CC cells and in β -thal cells from splenectomized patients was found to be markedly elevated but only slightly increased in β -thal cells from nonsplenectomized patients. Interestingly, when the total calcium content was measured in density fractionated cells from splenectomized β -thal patients, the highest calcium concentration was observed in the less dense fraction, indicating that they corresponded to cells normally removed by the spleen, ie, the most abnormal cells by a morphological criterion.²⁰

It is not clearly known at present how Ca^{2+} accumulates in these pathologic cells. In β -thal cells, previous reports have shown that Ca^{2+} influx was increased about 12-fold,¹⁰ whereas the Ca^{2+} , Mg^{2+} , ATPase activity was not modified.⁷ It was thus suggested, as now demonstrated in sickle cell anemia red cells,^{5,6} that Ca^{2+} might be trapped within endocytic vacuoles known to occur in β -thal cells,²⁰ making this extra Ca^{2+} unavailable for Ca^{2+} pump extrusion.⁷ The

Ca^{2+} turnover in CC cells has never been studied. However, the fact that their low K^+ , Na^+ , and water content was not a Ca^{2+} -dependent phenomenon¹¹ could indicate that, in CC cells also, the excess of Ca^{2+} is sequestered into internal vesicles.

In the present study, Ca^{2+} influx was measured after incorporation of a moderate concentration of Ca^{2+} chelator in order not to affect the metabolic state of the cells.¹⁷ In AA and CC cells and β -thal cells from nonsplenectomized patients, exchangeable Ca^{2+} behaved as one homogeneous pool, and Ca^{2+} influx into this pool was the same in pathologic cells and in AA cells (26 to $31 \mu\text{mol/L cells/h}$). This is in contrast to the results previously mentioned reporting an increase in Ca^{2+} influx in β -thal cells;¹⁰ these results, however, were obtained in ATP- or Mg^{2+} -depleted cells. The measurement of Ca^{2+} influx in the presence of the chelator, which leaves the activity of the Ca^{2+} pump unaltered, is likely to be a more physiological approach than the method involving the inhibition of this pump.

In β -thal cells from splenectomized patients, two exchangeable Ca^{2+} pools were observed. Ca^{2+} influx into the major pool, which contained about 80% of the exchangeable Ca^{2+} , was identical to Ca^{2+} influx into AA cells. Ca^{2+} influx into the minor pool ranged between 35 and $70 \mu\text{mol/L cells/h}$. In order to determine whether the presence of the minor pool was due to the presence of reticulocytes, which amounted to about 5% of the β -thal cell population, or to other cell populations that would otherwise have been removed by the spleen, experiments were carried out in AA cells from reticulocyte-rich patients or from splenectomized persons. In neither case was the kinetic of the ^{45}Ca uptake different from that in normal AA cells. However, when Ca^{2+} influx was measured in density-fractionated β -thal cells from splenectomized patients, it was found that the lightest fraction exhibited a kinetic heterogeneity, whereas the more dense cells had only one pool of exchangeable Ca^{2+} with a nearly normal Ca^{2+} influx. Thus, it can be concluded that in β -thal patients, splenectomy uncovers a population of light cells characterized by an elevated total calcium content and a slightly increased Ca^{2+} influx (up to twofold). In cells from nonsplenectomized β -thal patients, total calcium content is nearly normal, as is Ca^{2+} influx. Finally, CC cells represent an intermediate situation in which total calcium content is increased, whereas Ca^{2+} influx is normal.

The $[\text{Ca}]_i$ in these pathologic cells, which has not been determined before, was in the same range as that observed in AA cells (about 25 nM). This interesting finding brings further support to the suggestion that, as in sickle cells, the excess of Ca^{2+} in β -thal cells is not free in the cytoplasm but trapped within endocytic vacuoles. The presence of such vesicles has been observed in β -thal cells, particularly from splenectomized patients.²⁰ Thus, it seems that splenectomy in β -thalassemia or asplenia associated with sickle cell anemia could be the origin of the increase in the total calcium concentration. This could also be the case in CC cells. However, hemoglobin C patients have a functional spleen, and the persistence of such vesicle-containing cells is more questionable. It is possible, in this case, that a different defect is involved that would explain the calcium retention.

Although these pathologic cells are characterized by several membrane alterations,^{9,12} including cation permeability modifications and by a high calcium content, the present experimental evidence demonstrates that the Ca^{2+} permeability and the resting level of free cytosolic Ca^{2+} are normal.

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Note Added in Proof

While this paper was submitted, an abstract by Bookchin et al²¹ reported similar results on the state and transport of calcium in β -thal red cells.

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A δ -Globin Gene Derived From Patients With Homozygous δ^0 -Thalassemia Functions Normally on Transient Expression in Heterologous Cells

By Takanori Nakamura, Yoshihiro Takihara, Yoshiro Ohta, Shigeru Fujita, Yasuyuki Takagi, and Yasuyuki Fukumaki

Three Japanese individuals with homozygous δ^0 -thalassemia from different families were the subjects of molecular genetic analysis. They were homozygous for seven polymorphic sites in the β -globin gene cluster. Nucleotide sequence analysis of the δ -globin gene cloned from each patient revealed a single nucleotide substitution (T-C) 77 base pairs 5' to the cap site, just upstream of the CCAAC box of the δ -globin gene. When introduced into COS cells, the gene was expressed at normal levels with proper

processing of RNA. These results suggest that the complete suppression of δ -globin chain synthesis in these patients is not due to a defective promoter, a defective RNA processing or a chain terminator mutation, but rather to impaired regulation of gene expression specific to erythroid cells. The region around the CCAAC box may have a significant role in expression of the δ -globin gene in erythroid cells.

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THE THALASSEMIA is characterized by discordant rates of synthesis of structurally normal peptide chains resulting in a deficiency of hemoglobin. Depending on the chain involved, four types are presently known: α^- , β^- , δ^- , and $\delta\beta$ -thalassemia. A molecular genetic analysis of α^- , β^- , and $\delta\beta$ -thalassemias revealed various structural changes in globin genes such as deletions, nonsense mutations, frameshift mutations, promoter mutations, and mutations affecting RNA processing.^{1,2} Furthermore, functional studies, in which cloned genes are introduced into heterologous cells and then the transcribed RNAs are analyzed, have demonstrated the effects of mutations on gene expression.³⁻⁵

δ -Thalassemia is a rare condition and has been studied mainly in Italy,^{6,8} the USA,⁹ and Japan.¹⁰⁻¹⁴ The lesion responsible for the impairment of the δ -globin chain production in δ -thalassemia has not been defined.^{13,14} In an effort to characterize the molecular pathology of δ -thalassemia, we isolated δ -globin genes from three unrelated individuals with homozygous δ^0 -thalassemia and analyzed the nucleotide sequence and transcription of the cloned gene. We report here the identification of a single base change 77 base pairs upstream from the cap site of δ -globin genes derived from the homozygotes and quantitatively and qualitatively normal expression of one of these δ -globin genes introduced into the COS cells. The implications of these findings for the cell- or developmental stage-specific expression of the δ -globin gene are discussed.

MATERIALS AND METHODS

Subjects. We studied three Japanese patients with homozygous δ^0 -thalassemia (patients no. 1, 2, and 3). Hematological data are as follows: Patient no. 1, Hb 13.0 g/dL, Ht 41%, RBC 4.47×10^6 /mm³; patient no. 2, Hb 13.6 g/dL, Ht 41%, RBC 4.81×10^6 /mm³; patient no. 3, Hb 10.7 g/dL, Ht 31%, RBC 3.34×10^6 /mm³. On electrophoresis and DEAE-sephadex column chromatography of the hemoglobin, HbA₂ was absent. The level of HbF estimated by the method of alkali denaturation was 4.7%, being distributed heterogeneously in the red cells in patient no. 1, 1.0% in patient no. 2, and 0.9% in patient no. 3. Hematologic values for a sister of patient no. 2 were: Hb 12.6 g/dL, RBC 4.33×10^6 /mm³, Ht 35%, HbA₂ 1.4%, HbF 0.9%. The pedigree of each family was described in ref. 12. Patient no. 1 is II-4 of a family M. Patient no. 2 and a sister of Patient no. 2 correspond to II-4, and II-1 of a family O, respectively. Patient no. 3 is I-1 of a family IZ.

Restriction enzyme analysis. High molecular weight DNA was prepared from peripheral leukocytes of each patient as described.¹⁵ Restriction endonuclease digestion of genomic DNA and blot hybridization were performed as described.¹⁶ For analysis of seven

restriction site polymorphisms in the β -globin gene cluster¹⁷ (Fig 1), a 0.8 kb *Bam*HI fragment containing a ϵ -globin gene, a 3.4 kb *Hind*III A_γ -globin gene fragment, a 4.0 kb *Bgl*III $\psi\beta$ -globin gene fragment, and a 4.4 kb *Pst*I fragment containing a β -globin gene were used as probes.

Gene cloning and DNA sequencing. High molecular weight DNA from Patients no. 1 and 2 was partially digested with *Eco*RI and size-fractionated by sucrose gradient sedimentation. DNA fragments 15 to 20 kb in size were inserted into the *Eco*RI site of the lambda Charon 4A DNA.¹⁸ The δ -globin gene clones were isolated by plaque hybridization with a 2.3 kb fragment containing a normal δ -globin gene.¹⁹ A δ -globin gene clone of Patient 3 isolated previously¹³ was used as a source for DNA sequencing analysis. The δ -globin gene region was subcloned in pUC9 and sequenced by the dideoxy chain termination method.²⁰

Construction of the globin-SV40 recombinant molecules. Plasmid vector pSVori2172²¹ carrying the SV40 *Pvu*II-*Bgl*III fragment that contains the origin of DNA replication and the tandemly repeated 72 bp sequence was used as a vector for expression of the globin gene in COS cells.²² The 2.3 kb *Pst*I fragment containing the δ -globin gene with the 449 bp 5' flanking and the 207 bp 3' flanking region was cloned at the *Bam*HI site of pSVori2172, as shown in Fig 2.

Gene expression in COS cells. Twenty micrograms of normal δ -globin gene recombinants and the recombinants carrying the δ -globin gene isolated from the homozygous δ -thalassemia patient were added as calcium phosphate precipitates to each 100-mm culture dish of subconfluent COS monkey kidney cells²² separately with 2 μ g of the α -globin recombinant²³ as an internal reference. After 48 hours of incubation at 37°C, cells were harvested and RNA was prepared by lysis in guanidinium thiocyanate solution and by cesium chloride density centrifugation as described.²⁴ Probe excess-S1 nuclease mapping of the cellular RNAs was prepared using

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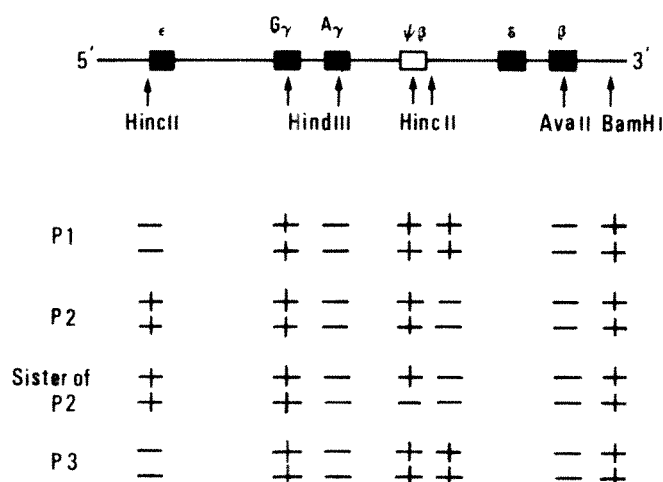


Fig 1. Haplotype of the β -globin gene cluster. The seven polymorphic restriction sites are shown in the β -globin gene cluster from patients with δ -thalassemia. (+) and (—) indicate the presence and absence of a restriction site, respectively.

appropriate probes labeled at either their 5' or 3' termini.²⁵ Hybridizations were performed overnight at 51°C. Hybrids were digested at 37°C for 30 minutes with 7 to 10 U/mL S1 nuclease purchased from Sankyo Company Ltd, Japan. Protected DNA fragments were electrophoresed in urea-acrylamide gels, which were subjected to autoradiography with intensification. The amounts of each RNA that were required to yield equivalent α -probe signals were first determined and then used to quantitate relative δ -globin RNA levels.

RESULTS

Restriction analysis of the β -globin gene cluster. The structure of the δ -globin gene region in high molecular weight DNA from each subject was analyzed by Southern blotting following digestion with a variety of restriction enzymes. Normal restriction digestion patterns were observed in the δ -globin gene region, indicating no possibility of gross rearrangement or deletion resulting in the δ -thalassemia phenotype (data not shown). To examine homozygosity for the globin allele of these patients, we analyzed the genomic DNA with respect to common DNA polymorphisms within the β -globin gene cluster.¹⁷ Patients no. 1 and 3 had the same haplotype of the β -globin gene cluster. The haplotype of patient no. 2 was different from that of patients no. 1 and 3. However, these three patients were homozygous at seven different polymorphic restriction sites, as shown in Fig 1. A δ -thalassemia trait, a sister of patient no. 2, had two different haplotypes, one of which was the same as that of patient no. 2. Homozygosity for one haplotype suggested that these patients were homozygous for a particular type of the δ -globin gene mutation. Thus, a single cloned δ -globin gene was isolated from each homozygote for further analysis.

Nucleotide sequence analysis of the cloned δ -globin gene. We determined the nucleotide sequence of the δ -globin gene region from 449 bp 5' to the cap site to 207 bp 3' to the polyadenylation site of the δ -globin gene isolated from each patient. Comparison of the nucleotide sequence of two homozygous patients with that of the δ -globin gene region published previously²⁶ revealed four sequence changes within the 5'-flanking region, a T-C change at position -77, a C-T change at position -199, an A addition in a 4-base stretch of adenosine residue located from positions -408 to -411, and

an insertion of an AT dinucleotide between positions -447 and -448 (Fig 3). The base substitutions at positions -77 and -199 of the δ -globin gene derived from patient no. 3 were not identified in the previous study.¹³ These discrepancies could be due to sequencing errors. We also determined the nucleotide sequence of the δ -globin genes isolated from two Japanese patients with no hematological abnormalities (N1, N2 in Fig 3). These data showed that the base changes in the 5' flanking region, except for a T-C change at position -77, were considered to be sequence polymorphisms because they were also found in the δ -globin genes of the normal subjects. (Fig 3). Therefore, a T-C change at position -77 is a common change among the δ -globin genes of the patients with homozygous δ^o -thalassemia and might have functional significance in δ -globin gene expression.

To eliminate the possibility that the thalassemia phenotype is due to a small deletion or rearrangement upstream from the δ -globin gene, we performed detailed restriction mapping of the 3.1 kb and 2.3 kb *EcoRI* fragments that were subcloned from the phage clone of Patient 2. The region approximately 3.9 kb upstream from the δ -globin gene is covered by these fragments. No differences were seen between the fragments derived from Patient 2 and the normal counterpart (data not shown).

Functional analysis of the cloned δ -globin gene. Assessment of the functional impairment of α - and β -thalassemia genes has been accomplished largely using transient expression in heterologous cells.³⁻⁵ For functional analysis of the δ -globin gene of patients with δ^o -thalassemia, we examined the function of this gene upon transient expression in COS cells from the viewpoint of transcriptional efficiency and processing of RNA. SV40 recombinants containing normal and patient δ -globin genes were transfected in parallel into subconfluent COS cells with the α -globin gene recombinant as an internal standard. Total cellular RNA was analyzed 48 hours after transfection by S1 nuclease mapping. In this expression system, the δ -globin RNA present in COS cells was about 1/50 as much as the α -globin RNA in COS cells transfected with the α globin-SV recombinants according to the intensity of corresponding bands in a S1 nuclease analysis, when standardized for differences in the specific activity of each probe and amount of recombinants used for transfection.

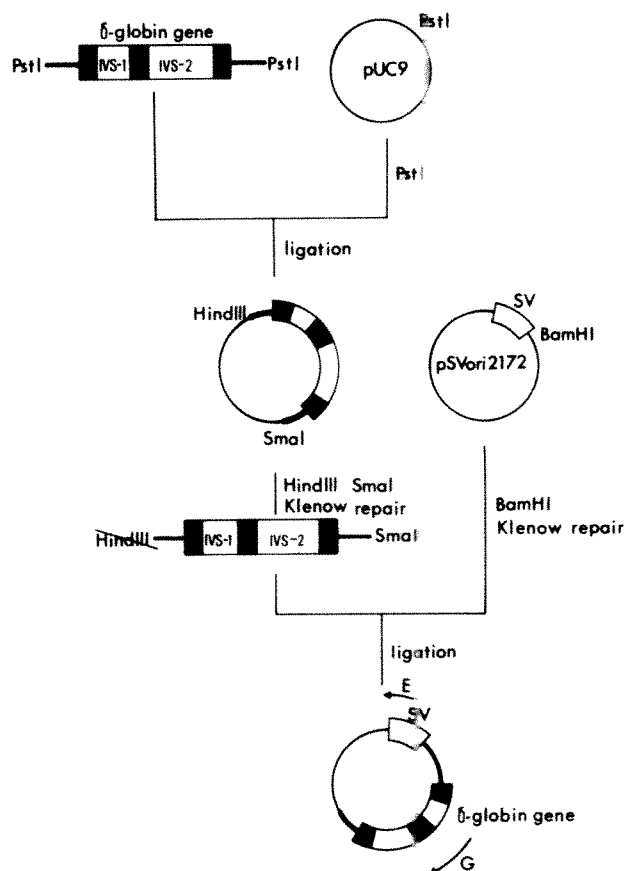


Fig 2. Construction of globin-SV40 recombinant molecules. SV: the SV40 *PvuII-BglII* fragment containing sequences necessary for efficient δ -globin gene expression in COS cells. *PstI* fragments containing the δ -globin gene were inserted into pUC9 and then the δ -globin gene fragment was excised from the recombinant by digestion with *SmaI* and *HindIII*. After repairing the *HindIII* site of δ -globin gene fragment with Klenow fragment (large fragment of DNA polymerase I), the δ -globin gene DNA fragment with both blunt ends was ligated to pSVori2172 linearized with *BamHI* followed by Klenow fragment repair at both termini. Polarity of SV40 transcription unit and δ -globin gene transcription unit is indicated by arrow with E and G, respectively.

tion. The S1 nuclease mapping after hybridization with two kinds of fragments as DNA probes showed that identically sized fragments corresponding to the part of the exon2 were protected and that the patient globin gene directed the synthesis of the δ -globin RNA almost as much as normal (Fig 4A). Further S1 nuclease mapping demonstrated no difference between expression of patient and normal genes in the splice site of IVS2, the initiation site of transcription, and the cleavage site for polyadenylation (Fig 4 B,C,D). There is no evidence from these studies to indicate that the patient δ -globin gene contains a mutation affecting RNA transcription or processing.

DISCUSSION

There were no apparent nucleotide changes within the coding region and IVS of the δ -globin gene isolated from three Japanese patients with homozygous δ° -thalassemia except for one base substitution (T-C) at position -77 in the

	5'	-448	-408	-276	-199	-77	IVS-1	IVS-2	3'
P1		AT	A	A	T	C			
P2		AT	A	A	T	C			
P3		AT	A	A	T	C			
N1		AT	A	G	T	T			
N2		AT	A	A	C	T			
N3		O	O	A	C	T			
N4		ND	ND	ND	ND	T			

Fig 3. DNA sequence of the globin gene of the normal subjects and patients with homozygous δ° -thalassemia. P1, P2, and P3 are the sequence of the δ -globin gene isolated from patients no. 1, 2, and 3, respectively. N1 and N2 are from normal Japanese subjects. N3 and N4 reveal the DNA sequence of the normal δ -globin gene reported by Poncz et al.²⁶ and Spritz et al.¹⁹ respectively. ND indicates the sequence not determined.

5' flanking region. Functional analysis of the patient δ -globin gene upon transient expression in COS cells demonstrated neither qualitative nor quantitative differences when compared to the reference δ -globin gene. The sequence and transcription data indicate that the phenotype observed in these patients is not due to a defective promoter, defective RNA processing, or a chain terminator mutation.

Recently two cases of thalassemia were reported in which the cloned β -globin genes expressed normally in heterologous cells.^{27,28} In one case,²⁷ a deletion of the entire gene cluster 2.5 kb 5' to the β -globin gene produced a $\gamma\beta$ -thalassemia phenotype. Although in this form of thalassemia the β -globin gene has a normal sequence and functions normally in heterologous cells, the affected β -globin gene is present in an inactive configuration in vivo. It is suggested that a normally inactive locus was translocated to the affected globin gene, or the sequences that are required for activating the gene were deleted. There was no evidence of a deletion or DNA arrangement in the β -globin gene cluster of patients studied here.

The other case was the silent carrier allele found in an Albanian family.²⁸ This case was not due to a mutation within the β -globin structural gene or its flanking regions. Furthermore, haplotype analysis revealed that the affected offspring inherited a different β -globin gene cluster from the father. Therefore, the silent carrier allele may not be linked to the β -globin gene cluster. There might be abnormalities in trans-acting factors that affect the expression of gene in the β -globin gene cluster. Three patients with homozygous δ° -thalassemia were homozygous for two kinds of haplotype. However, a crossing-over event in the region 5' to the β -globin gene can account for the existence of two different haplotypes associated with the base substitution at position -77 in homozygous patients. The region 10 kb 5' to the β -globin gene is thought to increase recombination in the β -globin gene cluster.²⁹ In fact, it is suggested that the β^S mutation spread from one haplotype to another by this type of recombination event.³⁰ The sister of patient no. 2 with heterozygous δ -thalassemia inherited the haplotype possessed by patient no. 2 and another haplotype that was not observed in three homozygous patients studied here. These results suggest that the δ -thalassemia locus is linked to the

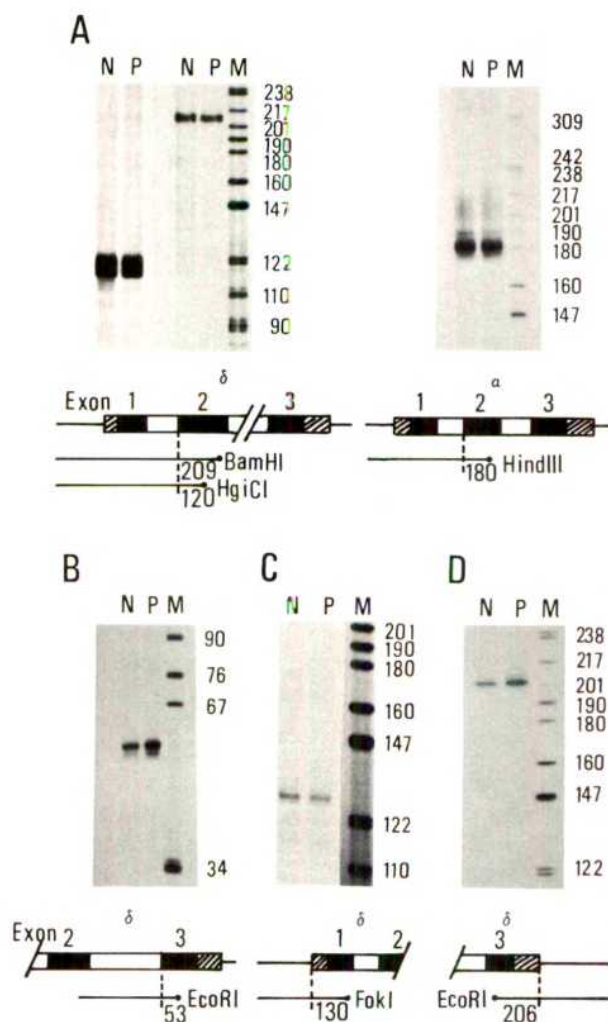


Fig 4. Expression of the patient δ -globin gene in COS cells. The normal (N) and the patient globin-SV40 recombinants (P) were separately cotransfected with the normal α -globin-SV40 recombinants into COS cells. Total cellular RNA was purified as described in Materials and Methods. Probe excess S1 nuclease mapping was performed using either the 5' or the 3' end-labeled DNA probes depicted at the bottom. (A) Mapping of the splice site of IVS1. The probes used were a 937 nt (nucleotides) *Pst*I-BamHI, a 848 nt *Pst*I-HgiCI δ -globin gene fragments and a 1.0 kb *Pst*I-HindIII α -globin gene fragment. (B) Mapping of the splice site of IVS2. The probe was a 966 nt *Bam*HI-EcoRI δ -globin gene fragment. (C) Mapping of the cap site. The probe was a 588 nt *Pst*I-FokI δ -globin gene fragment. (D) Mapping of the polyA site. The probe was a 415 nt *Eco*RI-*Pst*I δ -globin gene fragment. Lengths of ^{32} P end-labeled pBR322 *Hpa*II marker fragments are indicated (M).

β -globin gene cluster, especially one base substitution at position -77 of the δ -globin gene. However, the possibility that the -77 mutation is a polymorphism in linkage disequilibrium with the δ -thalassemia determinant, and the true locus is located in another region, cannot be excluded.

The functional consequences of this substitution remain speculative. DNA sequences required for the transcriptional selectivity are close to or within structural sequences, at least in some cases such as immunoglobulin heavy and light chain genes,^{31,32} mammalian insulin genes,³³ a rat chymotrypsin

gene,³³ a rat elastase gene,³⁴ and a human α 1-antitrypsin gene.³⁵ With the exception of immunoglobulin genes, the upstream regulatory element is clearly required for expression in appropriate differentiated cells. The involvement of sequences 5' to the γ -globin genes in hemoglobin switching was postulated, based on a point mutation found at position -202 of the over-expressed G_{γ} -globin gene in G_{γ} HPFH.³⁶ In support of this hypothesis, an individual with the Greek type of HPFH and a southern Italian with a phenotype similar to Greek HPFH have been found to carry a point mutation at -117^{37,38} and -196³⁹ of the A_{γ} -globin gene, respectively. Particularly, a point mutation at -117 of inappropriately expressed A_{γ} -globin gene in adult life suggests that the CCAAT box (or its surrounding sequences) has a role in developmental control of γ -globin genes.³⁸

Recently Emerson et al⁴⁰ identified a positive factor in 9- to 14-day and adult chicken erythrocytes that interacts with the region extending -40 to -200 nucleotides from the cap site of the chicken adult β -globin gene and induces DNA hypersensitivity around the region, detected in only a transcriptionally active β -globin gene in vivo. These results indicate that a DNA region -300 bp 5' to the globin gene is important in a cell- and developmental stage-specific expression of the globin gene.

The nucleotide substitution shared by three independent patients with homozygous δ^0 -thalassemia is located 9 bp 5' to the degenerated CCAAT box, the CCAAC box of the δ -globin gene. The region around the CCAAC box of the δ -globin gene might be the binding site of a positive trans-acting factor normally present in adult cells. The one-base substitution at position -77 may inhibit the DNA-protein interaction, hence turning off expression of the affected gene. Functional studies of this δ -globin gene in erythroid cells under developmental control of its expression are in progress.

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Phenotypic Similarities and Differences Between CALLA-Positive Acute Lymphoblastic Leukemia Cells and Normal Marrow CALLA-Positive B Cell Precursors

By Daniel H. Ryan, Charles W. Chapple, Stuart A. Kossover, Avery A. Sandberg, and Harvey J. Cohen

Expression of differentiation markers in common acute lymphoblastic leukemia (cALL) cells from 25 patients was compared with subpopulations of normal common ALL antigen (CALLA) (CD10)-positive bone marrow lymphoid cells (cBMLs). In cBML, CD10 intensity is positively correlated with CD34 (MY10) and terminal transferase (TdT) expression and inversely correlated with common leukocyte antigen (CLA), CD20 (B1), and cytoplasmic μ chain ($C\mu$) expression. In cALL, CD10 density was inversely correlated with CLA and $C\mu$ expression and strongly correlated with CD34 expression as in cBML. In contrast to cBML, TdT and CD20 expression were not related to CD10 density in cALL. Furthermore, cALL TdT intensity measured by enzyme immunoassay was not related to expression of CD10, CLA, or CD34, but was positively correlated with CD20 expression. $C\mu$ expression in cALL was inversely correlated with expression of CD34 and positively correlated with CLA as in cBML, but showed no

association with TdT intensity or CD20 expression in contrast to the relationship found in cBMLs. Analysis of TdT intensity and $C\mu$ expression in sorted subpopulations of cells from individual patients that were positive or negative for CD34, CD20, or CD10 was consistent with the data obtained by comparison of cells from different patients. These results indicate that from patient to patient and within individual patients, cALL cells express the markers CD34, CLA, CD10, and $C\mu$ in a coordinated fashion similar to cBMLs, but demonstrate differences in expression of TdT and CD20 with respect to the marrow cells considered their normal counterparts. The cALL cells that are CD34 positive show increased expression of CD10 and are less likely to be CLA or $C\mu$ positive, suggesting that they may represent a phenotypically less differentiated form of cALL than does CD34-negative cALL.

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EARLY B CELL precursors most likely originate in the bone marrow.¹ The malignancy that phenotypically most closely resembles these early stages of B cell maturation is acute lymphoblastic leukemia (ALL), a neoplasm that also usually arises in the bone marrow. The B cell origin of leukemic cells from most patients with ALL is suggested by the presence of immunoglobulin gene rearrangements.² These cells also express surface markers specific to the B cell lineage, such as CD19 (B4) and CD20,^{3,4} as well as markers characteristic of immature lymphoid cells, such as TdT and CD10. The most frequent ALL phenotype is CD10+/TdT+/CD19+ (common ALL), found in 75% of patients with ALL.⁵

Cells with the CD10+/TdT+/CD19+ phenotype are easily detected in children's marrow, and can be found in small numbers (1% to 5%) in adult bone marrow. We previously described two subpopulations of normal CD10-positive marrow lymphoid cells (cBMLs).⁶ The majority of these cells are weakly CD10+/CD20+/CLA+/CD34-/TdT-, whereas ~12% of the cells are strongly CD10+/

CD20-/CLA-/CD34+/TdT+. The phenotype of the former subpopulation more closely resembles that of mature B cells, suggesting that this is the more differentiated B cell population. Both CD10-positive marrow lymphoid populations express CD19 and lack CD5 (Leu1), suggesting that they belong to the B cell lineage. A recent study has confirmed these observations.⁷

The aim of this study was to compare the phenotype of cALL cells with the two subpopulations of cBML. The phenotypic resemblance of cALL to the total population of cBMLs⁸ suggests that on more detailed examination, cALL subpopulations like those in normal marrow may be present. Multiparameter analysis of cells from 25 patients with cALL demonstrates that expression of CD10, CD34, CLA, and $C\mu$ are correlated with each other in the same fashion as observed in cBML. In contrast, TdT and CD20 expression in cALL are unrelated to expression of the other differentiation markers. These data suggest that differentiation stages of B cell lineage cALL are present, but that differences are observed when cALL cells are compared in detail with the marrow CD10-positive lymphoid cells believed to be their normal counterparts.

MATERIALS AND METHODS

Patients. Bone marrow aspiration from the posterior iliac crest was performed at the time of diagnosis or relapse in 25 patients with cALL. The leukemic cells were peroxidase negative and CD10 positive with lymphoid morphology in all patients. In some patients, peripheral blood samples at the time of diagnosis were obtained if insufficient cells were obtained from the bone marrow aspiration for multiple marker studies and if the peripheral blood mononuclear fraction contained >80% lymphoblasts. Bone marrow aspiration from the posterior iliac crest was also performed on healthy adult donors after informed consent was obtained (n = 8). Samples from diagnostic bone marrow aspirates were obtained from children with the following diagnoses: immune thrombocytopenia (1), anemia (1), neuroblastoma not involving the bone marrow (1), and ALL in complete clinical remission (2). Collection of samples was performed

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after approval by the institutional Committee on Investigations Involving Human Subjects and in accord with an assurance filed with and approved by the Department of Health and Human Services.

Cell preparation. Bone marrow was collected in 10-mL sterile siliconized tubes containing 5 mL RPMI 1640 tissue culture medium, 10% fetal calf serum (FCS) and 100 U of preservative-free heparin. Peripheral blood was collected in sterile heparinized tubes. Ficoll/Hypaque separation of these cells was performed as described previously.⁹ Cells from patients with cALL were frozen in liquid nitrogen⁹ for later analysis.

Antibodies. Monoclonal antibodies were obtained from Becton Dickinson Monoclonal Center (Mountain View, CA) (anti-CALLA [CD10] FITC, anti-HLA-DR FITC, MY10 [CD34], avidin PE, FITC- and PE-conjugated irrelevant murine IgG1, and FITC-conjugated irrelevant murine IgG2), Coulter Immunology (Hialeah, FL) (B1 [CD20] FITC, B2 [CD21] FITC, B4 [CD19], J5 [CD10] biotin, biotin-conjugated irrelevant murine IgG2), PL Biologicals (anti-TdT), Southern Biotechnology (isotype specific goat anti-mouse IgG1/FITC), and Vector Biochemicals (peanut agglutinin [PNA] FITC). GAP 8.3 (anti-common leukocyte antibody) was obtained from the American Type Culture Collection and conjugated to PE as previously described.⁹

Immunofluorescence staining. The choice of anti-CD10 reagents (anti-CALLA FITC or J5 biotin followed by avidin PE) was based on the availability of FITC or PE conjugates of the second marker to be studied. All incubations with antibody were at 4°C, followed by three washes in phosphate-buffered saline (PBS). In each case, cells were washed three times in PBS and resuspended in 300 μ L PBS at 4°C for analysis on the flow cytometer.

For two-color immunofluorescence (IF) of CD10 v HLA-DR, common leukocyte antibody (CLA), B1 (CD20), B2 (CD21), PNA, B4 (CD19), and MY10 (CD34), cells were stained with monoclonal antibodies and control reagents as previously described.⁶

For combined B1 (CD20) and MY10 (CD34) staining of leukemic cells, the cells were incubated with appropriate dilutions of biotin-conjugated CD20 plus MY10 (CD34), followed by three washes and incubation with avidin PE plus isotype specific goat anti-mouse IgG1/FITC. Cells were stained with biotin-conjugated irrelevant murine IgG2 plus MY10 (CD34), followed by avidin PE plus isotype-specific goat anti-mouse IgG1/FITC to control for the B1 (CD20) staining. Cells were also stained with biotin-conjugated B1 (CD20) plus irrelevant murine IgG1 (MOPC 21) followed by avidin PE plus isotype specific goat anti-mouse IgG1/FITC to control for MY10 (CD34) staining.

Flow cytometry analysis. Samples were analyzed either fresh or after fixation with 1% paraformaldehyde. Stained cells were analyzed on an EPICS C flow cytometer using instrument settings as previously described.⁶ Forward-angle light scatter (FALS) gates were set to exclude RBCs, dead cells, platelets, or debris as well as large aggregates. Ninety-degree light scatter (L90LS) gates were set to exclude a distinct myeloid population with high L90LS signal when normal bone marrow was analyzed but were left open for analysis of cALL cells. Fifty thousand cells were counted per sample. A two-parameter 64×64 -channel histogram of log green fluorescence (LGFL) v log red fluorescence (LRFL) gated on FALS (or FALS and L90LS) was collected.

Data analysis. Data were analyzed using the EPICS C data analysis software (projection analysis software), which enables graphic analysis of the green fluorescence of cells showing a given range of red fluorescence and vice versa. Thus, cells positive for one antibody (eg, CD10) can be analyzed for staining with another antibody. For determination of the percentage of cells positive for a differentiation marker, only cells positive for the CD10 antigen were analyzed. A cutoff point was set at a point above which only 5% of

the CD10-positive cells were stained with the appropriate control antibody. The percentage of cells positive for each differentiation marker was determined as the proportion of CD10 positive cells above this cutoff.

To determine the fluorescence intensity of a stained population, the logarithmic fluorescence channel intensity was converted to arbitrary linear units based on determination that an increase of 6.25 log channels in the 64-channel two-parameter histogram is equivalent to doubling of the fluorescence intensity. This factor was determined using microspheres of calibrated fluorescence intensity.¹⁰ The linear intensity thus determined was adjusted for instrument variability by comparison with the fluorescence intensity of standard fluorescent microspheres analyzed on that day.

CD10 density on the cell surface was estimated using the FALS signal, which is proportional to cell size in cells of similar structure.¹¹ Since surface area is proportional to the two-thirds power of volume, the CD10 intensity was divided by the FALS intensity to the two-thirds power to estimate CD10 density. The correlation of CD10 intensity or density was used. CD10 density was estimated primarily to compare CD10 expression of cALL and cBML.

Cell sorting. For cell-sorting experiments, only fresh cells were used. Three-droplet sorting without coincidence correction at a flow rate of 800 cells/s was performed at a 32-kHz droplet formation rate. The sorting logic was based on FALS, 90° light scatter (for sorting experiments with normal bone marrow to gate out weakly CD10-positive neutrophils) and red and green fluorescence. Cells were sorted into a 1.5-mL conical polypropylene vial containing 0.5 mL McCoy's tissue culture medium with 10% FCS. The sorted cells were divided into aliquots for immunoperoxidase staining, TdT immunofluorescence, cell cycle analysis, and TdT enzyme immunoassay.

Immunoperoxidase staining. Cytoentrifuged cells were fixed in formal-buffered acetone (200 mg/L Na_2HPO_4 and 200 mg/L KH_2PO_4 in 45% acetone and 25% concentrated formalin) for 30 seconds at room temperature. The fixed slides were incubated for 30 minutes at room temperature with goat serum to reduce nonspecific binding. After being washed with PBS, slides were stained with an appropriate dilution of peroxidase conjugated goat anti-human μ heavy chain or goat anti-rabbit γ heavy chain (Tago Diagnostics, Burlingame, CA) as control for 30 minutes at room temperature, followed by washing in PBS and a 30-minute incubation with freshly prepared developing solution (270 mg/L 3-amino-9-ethylcarbazole and 0.03% hydrogen peroxide in 0.1 mol/L acetate buffer-pH 5.2, AEC). After being washed in PBS, slides were mounted in Aquamount, kept in the dark at 4°C, and viewed under a microscope within 4 hours of staining. The control preparation showed <1% positively stained cells.

TdT immunofluorescence. Detection of TdT antigen in single cells by indirect immunofluorescence was performed on methanol-fixed cytoentrifuge slides as described.⁶

TdT enzyme immunoassay. TdT antigen was quantitated by enzyme immunoassay (Abbott TdT-EIA kit, Abbott Laboratories, North Chicago) as described.¹² Cells (35,000) were frozen for 12 hours at -10°C in phosphate buffer and added to a microwell containing a microbead coated with goat anti-calf TdT antibody. After 90-minute incubation at 30°C, the beads were washed in distilled water, and rabbit anti-calf TdT-horseradish peroxidase conjugate was added to each well. After 90-minute incubation at 30°C, the beads were washed and incubated with *O*-phenylene diamine/ H_2O_2 substrate solution for 30 minutes at room temperature. The enzymatic reaction was terminated by addition of 1.0 mL 5% H_2SO_4 , and the absorbance was read at 492 nm on a Quantum 1 spectrophotometer (Abbott Laboratories). The TdT antigen concentration in the sample was calculated from a standard curve gener-

ated by analysis of standards containing 0, 5, 10, and 15 ng TdT/mL. The TdT antigen content per cell was calculated based on the initial cell count in the analyzed sample prior to freezing.

Cell cycle analysis. Cellular DNA content was determined by the method of Krishan.¹³ Sorted cells were centrifuged at 400 g and vigorously resuspended in Tris-PI solution [1.21 g/L Trizma base, 480 mg/L MgCl₂, 0.1% Triton X-100, 50 mg/mL propidium iodide, and 2 mg/mL RNase A (Sigma, St Louis)]. After at least 1 hour of incubation at 4°C, the cells were vigorously resuspended by 25 to 40 aspirations in a Pasteur pipette and analyzed on an EPICS C flow cytometer. The percentage of cells in S+G₂/M phases of the cell cycle was calculated by integrating the region of the DNA histogram (linear red fluorescence reflected from a 560 dichroic filter and collected through a 575-band pass filter) above the G₀/G₁ peak. At least 5,000 to 10,000 nuclei were counted for each sample. Cell cycle analysis was performed on sorted cALL cells; hence, no admixture of normal cells was present.

Combined immunofluorescence and immunoperoxidase staining. For detection of cytoplasmic μ heavy chain (C μ) in TdT-positive cells, a combined immunofluorescence/immunoperoxidase procedure was performed. Cells were cytocentrifuged and fixed in formol-buffered acetone as described above, then stained with rabbit anti-calf TdT followed by FITC-conjugated goat anti-rabbit Ig. After the final wash, cells were incubated with normal goat serum for 30 minutes and stained with peroxidase-conjugated goat anti- μ heavy chain as described above for immunoperoxidase staining. The percentages of TdT-positive and C μ -positive cells were similar to those observed with separate staining for these antigens.

RESULTS

Phenotype of cALL cells. Ficoll/Hypaque separated cells from bone marrow or blood of 25 patients with cALL were frozen in liquid nitrogen for phenotypic analysis. The proportion of leukemic cells in the samples was at least 80%, and usually >90%. Two-color immunofluorescence was used to identify differentiation antigens specifically on CD10-positive cells. To evaluate the precision of CD10 intensity measurement by immunofluorescence, multiple aliquots of cells (n = 10) from a single patient with cALL were thawed from liquid nitrogen storage and stained on different days with anti-CD10 FITC over the course of 3 months. The mean calculated CD10 intensity was 113 (arbitrary linear fluorescence intensity units) with an SE of 6.3. Similarly, precision of FALS measurement was determined by measuring FALS of aliquots (n = 10) of cells from a single patient with cALL over the course of 3 months. The mean FALS intensity was 357 (arbitrary linear intensity units) with an SE of 11.7. Staining for CD10 was performed multiple times on each cALL sample, yielding consistent results.

The percentage of cells positive for differentiation antigens, CD10 density, and TdT intensity (as measured by enzyme immunoassay) for leukemic cells from each patient are listed in Table 1. Adult patients with cALL had significantly higher numbers of CD34-positive cells (mean

Table 1. Expression of Differentiation Antigens in cALL

Patient No.	CD10 Density (MFI)	Cells Positive (%)										TdT Intensity (fg/cell)
		CD10	CD34	PNA	CLA	C μ	CD20	TdT	CD21	CD19	Ia	
1	106	95	100	21	21	1	100	60	3	100	100	35
2	20	94	0	98	99	5	6	94	0	79	98	17
3	37	81	5	60	35	84	22	69	15	100	96	1
4	5	74	0	0	95	89	0	0	0	100	88	0
5	12	96	0	93	6	83	35	70	8	99	98	27
6	79	97	70	10	5	0	60	95	8	92	89	12
7	106	85	98	99	18	6	100	58	49*	96	97	45
8	39	91	85	0	70	44	40	83	0	95	97	26
9	12	83	4	97	3	2	2	90	2	99	97	18
10	92	84	74	46	0	4	7	97	0	100	ND	17
11	130	80	99	0	7	0	18	83	1	99	74	23
12	125	86	72	30	10	69	95	95	17	99	ND	69
13	56	84	48	0	5	0	89	85	40*	95	ND	31
14	61	85	30	12	3	ND	10	ND	4	80	ND	ND
15	32	89	7	84	9	0	7	88	0	99	99	25
16	8	86	19	0	7	36	34	77	0	99	ND	12
17	12	96	8	12	2	43	24	79	12	99	ND	12
18	180	40	99	0	2	0	19	77	2	94	ND	11
19	13	46	0	1	10	37	10	62	0	70	ND	26
20	169	93	50	10	8	7	2	91	44*	91	ND	15
21	103	63	91	4	0	0	63	72	17	93	96	24
22	22	86	21	85	1	2	58	86	2	90	89	58
23	32	95	3	77	0	6	12	71	43*	83	82	13
24	7	50	0	100	95	55	13	56	0	99	78	0.2
25	121	85	66	78	11	2	69	67	0	99	98	15
cALL Mean	63	82	42	41	21	24	36	75	11	94	92	22
cBML Mean	16	100	34	56	59	33	50	18	5	85	90	ND

Abbreviations: PNA, peanut agglutinin; CLA, common leukocyte antigen; C μ , cytoplasmic μ heavy chain; TdT, terminal transferase; ND, not done; MFI, mean fluorescence intensity (arbitrary linear units).

*Very weakly positive; cBML data from ref 9.

72% $\pm$ 17% SEM; $n = 6$) than children (mean 30% $\pm$ 8%; $n = 19$). No other significant phenotypic difference between these groups was demonstrated. Patients studied at relapse had significantly higher numbers of PNA-positive cells (mean 70% $\pm$ 17%; $n = 6$) than did patients studied at first diagnosis (mean 30% $\pm$ 8%; $n = 19$). No significant correlation was observed between antigenic phenotype and other clinical parameters (age, initial leukocyte count, sex, cytogenetics, survival) in this group of patients.

The mean expression of most differentiation antigens by cALL cells rather closely resembled cBML (Table 1), with the exception of TdT (more consistently expressed in cALL) and CLA (higher expression in normal marrow). The phenotype of cBML was obtained from data previously published,⁶ with the exception of C μ , which was demonstrated in 32.0% of sorted CD10-positive cells from normal bone marrow ($n = 6$) using the sensitive immunoperoxidase technique.

CD10 expression in cALL and normal marrow. Patients with cALL were grouped according to CD10 intensity as was previously done for normal marrow CD10 positive cells.⁶ A level of 50 fluorescence intensity units for CD10 density was used to divide the cALL patients into two nearly equal groups. Figure 1 shows the expression of differentiation

markers by strongly v weakly CD10-positive cALL as compared with strongly v weakly CD10-positive cBML. Weakly CD10-positive cALL was significantly more likely to be CLA- or C μ -positive but less likely to be CD34-positive or CD20-positive than strongly CD10-positive cALL. A trend toward higher PNA activity in weakly CD10-positive cALL did not reach statistical significance. No statistically significant difference in the percentage of TdT positive cells was demonstrated in the two populations of cALL. These findings are similar to those observed in strongly v weakly CD10-positive cBML (Fig 1B), with the notable exception of CD20 and TdT. Figure 1B is derived from data previously published,⁶ with the exception of C μ expression.

TdT expression in cALL and normal marrow. TdT expression is strongly correlated with CD10 intensity in

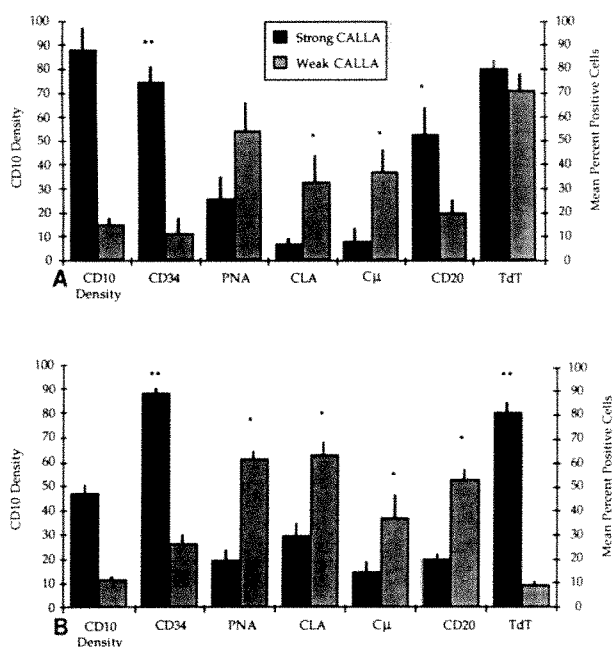


Fig 1. Comparison of weakly ($n = 13$) v strongly ($n = 12$) CD10-positive cALL (A), or weakly v strongly CD10-positive cBMLs ($n = 6$ to 9) (B). Antigen positivity (except TdT and C μ) of CD10-positive cALL or cBMLs was determined by two-color immunofluorescence. TdT and C μ were measured on cytocentrifuged cALL cells or cBMLs sorted on the basis of CD10 intensity. Data shown in panel B were previously published⁶ and are shown here for comparison. Ordinate shows the mean percentage of cells positive for each antigen indicated on the abscissa. For CD10, the ordinate represents the mean CD10 density of the weakly or strongly CD10-positive populations. Error bars = SEM. Confidence limits (* $P < .05$; ** $P < .001$) for the difference between weakly and strongly CD10-positive cells were calculated using the paired t test (cBMLs) or the unpaired t test (cALL).

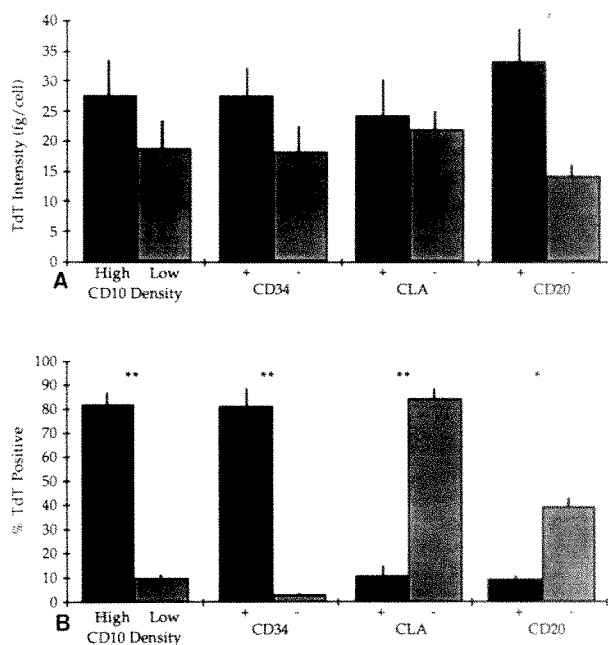


Fig 2. (A) TdT expression in cALL. Ordinate shows TdT intensity measured by enzyme immunoassay. Solid columns: Patients positive for antigen indicated on abscissa or displaying high CD10 density. Stippled columns: Patients negative for antigen indicated on abscissa or displaying weak CD10 density. Patients were considered positive for an antigen if at least 30% of the CD10-positive cells were positive for that antigen. Patients were considered strongly CD10 positive if the leukemic cells displayed CD10 density of at least 50 mean fluorescence intensity units (MFI). (B) TdT expression in sorted subpopulations of cBMLs ($n = 5$). TdT positivity of CD10, CD34, CD20, and CLA subpopulations of CD10-positive cells was determined by sorting for TdT immunofluorescence staining on cytocentrifuge preparations. Ordinate shows percentage of TdT-positive cells. Solid columns: Sorted CD10-positive cells positive for antigen indicated on abscissa or displaying high CD10 density. Stippled columns: Sorted CD10-positive cells negative for antigen indicated on abscissa or displaying low CD10 density. The cutoff for strong CD10 positivity was selected to best distinguish the two subpopulations evident in normal marrow and was ~25 fluorescence intensity units. Error bars = SEM. Confidence limits (* $P < .05$; ** $P < .001$) for the difference between TdT expression of antigen-positive and antigen-negative cells were calculated using the paired t test (cBML) or the unpaired t test (cALL).

normal marrow.⁶ Examination of TdT immunofluorescence preparations revealed that although nearly all the cALL cells were TdT positive, intensity of TdT expression was quite variable, as previously demonstrated.¹⁴ TdT intensity was measured by enzyme immunoassay to detect quantitative changes in TdT expression that might be related to expression of other differentiation markers. As shown in Fig 2A, there was no significant difference in TdT expression between strongly and weakly CD10-positive cALL, CLA-positive and CLA-negative cALL, and CD34-positive and CD34-negative cALL. CD20-positive cALL showed more intense TdT expression than did CD20-negative cALL. This pattern is in contrast to cBMLs, which show a strong correlation between CD34 positivity or CD10 intensity and TdT positivity and a strong inverse correlation between CLA or CD20 positivity and TdT positivity (Fig 2B).

Cytoplasmic μ expression in cALL and normal marrow. As shown in Fig 3A, there was no significant difference in $C\mu$ expression between CD20-positive and CD20-

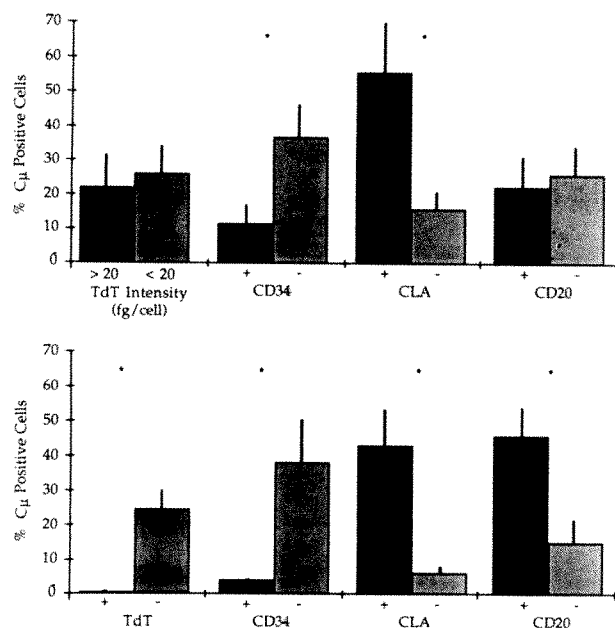


Fig 3. (A) $C\mu$ expression in cALL. Ordinate shows percentage of $C\mu$ -positive cells. Solid columns: Patients positive for antigen indicated on abscissa or displaying strong TdT intensity. Stippled columns: Patients negative for antigen indicated on abscissa or displaying weak TdT intensity. Patients were considered positive for an antigen if at least 30% of the CD10-positive cells was positive for that antigen. Patients were considered strongly TdT positive if the leukemic cells displayed TdT intensity of at least 20 fg/cell. (B) $C\mu$ expression in sorted subpopulations of cBMLs ($n = 5$). $C\mu$ positivity of TdT-positive and TdT-negative cells was determined by combined TdT immunofluorescence and $C\mu$ immunoperoxidase on sorted CD10 positive cells. $C\mu$ positivity of CD34, CD20, and CLA subpopulations of CD10-positive cells was determined by sorting these subpopulations for $C\mu$ -positive cells. Solid columns: Sorted CD10-positive cells positive for antigen indicated on abscissa. Stippled columns: Sorted CD10-positive cells negative for antigen indicated on abscissa. Error bars = SEM. Confidence limits (* $P < .05$; ** $P < .001$) for difference between TdT expression of antigen-positive and antigen-negative cells were calculated using the paired t test (cBML) or the unpaired t test (cALL).

negative cALL or between strongly TdT-positive and weakly TdT-positive cALL. However, CLA expression was correlated with $C\mu$ expression and CD34 positivity was inversely correlated with $C\mu$ expression in cALL. The pattern of CD20 and TdT reactivity in cALL is in contrast to cBMLs, which show a strong correlation of both CLA and CD20 positivity with $C\mu$ expression and a strong inverse correlation of both CD34 and TdT positivity with $C\mu$ expression (Fig 3B). The relationship of $C\mu$ and TdT expression in normal marrow was directly assessed by combined staining for $C\mu$ and TdT in sorted CD10-positive cells, as described in the Materials and Methods section. Although both TdT-positive (mean $19.4\% \pm 4.3\%$) and $C\mu$ -positive (mean $19.0\% \pm 5.1\%$) cells were found in sorted CD10-positive populations ($n = 5$), cells positive for both TdT and $C\mu$ were extremely rare (0.025% of CD10-positive cells).

Subpopulations of cALL cells in individual patients. Subpopulations of cALL cells within individual patients were observed with respect to CD34, CD20, and CD10 positivity. Figure 4 shows examples of patients with subpopulations of leukemic cells positive and negative for CD34 (Fig 4A and B), CD20 (Fig 4C and D), and CD10 (Fig 4E and F). These subpopulations were studied further to

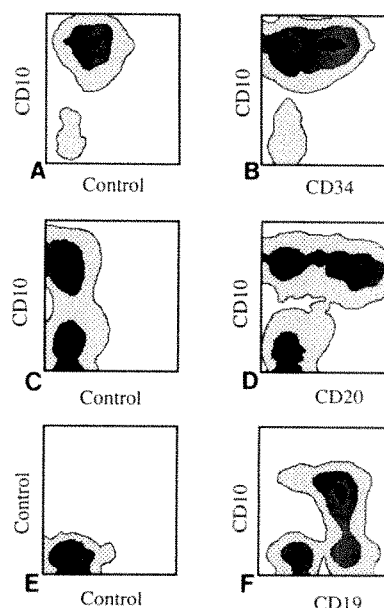


Fig 4. Subpopulations of CD34 (A), CD20 (B), and CD10 (C) positive and negative cells in three individual patients with cALL. Samples were stained with an anti-CD10 monoclonal antibody (J5 biotin) plus another marker or control as described in the Materials and Methods section. In all histograms, CD10 intensity is represented on the ordinate and the other marker or control is represented on the abscissa. For each histogram, 50,000 cells were counted. The contour lines indicate increasing number of cells in a given channel of the 64×64 -channel histograms. (A) Control v CD10, (B) CD34 v CD10, (C) control v CD10, (D) CD20 v CD10, (E) control v control, and (F) CD19 v CD10. Both CD34-positive and CD34-negative subpopulations of CD10-positive cells are apparent in B, and both CD20-positive and CD20-negative subpopulations of CD10-positive cells are apparent in D. Both CD10-positive and CD10-negative subpopulations of cells staining with the B cell-specific marker CD19 are present in F.

determine if consistent differences in expression of differentiation antigens could be identified. CD10 and FALS intensity were determined at the time of analysis on the flow cytometer, as described in the Materials and Methods section. Sorted cells were centrifuged and stained with propidium iodide for reanalysis on the flow cytometer to evaluate cell cycle kinetics of the subpopulations. Cytoentrifuge preparations of sorted cells were stained for C μ using an immunoperoxidase technique and for TdT using immunofluorescence. Finally, sorted cells were frozen at -10°C for quantitative measurement of TdT antigen by enzyme immunoassay. Table 2 shows the means and significance levels for differences in each of these parameters between the pairs of sorted subpopulations. CD34-positive cells were slightly larger and showed significantly higher CD10 density and lower C μ expression than CD34-negative cells from the same patient. CD20-positive cells showed no difference in CD10 density or C μ expression as compared with CD20-negative cells from the same patient, but were larger and did demonstrate a slightly lower TdT intensity. CD10-positive cells showed no difference in C μ expression or TdT intensity compared with CD10-negative cells from the same patient. Significant differences in cell cycle kinetics or percentage of TdT-positive cells by immunofluorescence were not found in any of the groups.

Phenotypic studies of ALL suggest that CD10 is absent on the most immature subtype of ALL,⁸ the CD19+/TdT+/CD10- ("null") ALL as well as on more mature B cell neoplasms. Thus, the sorted CD10-negative cells may be either more or less mature cells than the CD10-positive cells. These cells did not represent residual normal cells, since they were TdT positive, as were the CD10-positive cells from the same patients. We therefore subdivided the patients in whom we sorted CD10-negative and CD10-positive cells into patients in whom the CD10-negative cells demonstrated some degree of CD34 positivity ($n = 2$) as a sign of immaturity¹⁵ and patients in whom the CD10-negative cells were CD34 negative ($n = 2$). The CD10-negative cells of patients with CD10-/CD34- cells demonstrated higher C μ positivity (55% and 64%) than the CD10-positive cells (11% and 15%, respectively). The CD10-negative cells of patients with CD10-/CD34+ cells demonstrated lower C μ positivity (29% and 3%) than the CD10-positive cells (58% and 32%, respectively).

In six patients with both CD34 and CD20 subpopulations combined staining for CD34 and CD20 was performed, as described in the Materials and Methods section. In five of the six patients, the CD34-positive cells were as likely to be

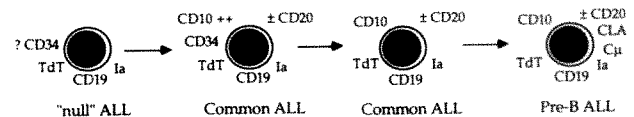


Fig 5. Hypothesis of differentiation-related subtypes of B cell lineage ALL.

CD20 positive as were the CD34-negative cells. In only one patient did the CD34 and CD20 antigens identify separate subpopulations of cells.

DISCUSSION

The CD10-positive lymphoid cell occurring in normal bone marrow has a close phenotypic similarity to CD10-positive ALL.¹⁶⁻¹⁹ Although CD10-positive cells have been described in kidney,²⁰ thymus,²¹ and lymph node,²² CD10+/TdT+/CD19+ cells (ie, the cALL phenotype) are confined to bone marrow. This suggests that the cell of origin in cALL may be closely related to the normally occurring bone marrow CD10 positive lymphocyte (cBML). We recently identified two phenotypic subpopulations of cBMLs.⁶ The phenotype of a minority population of strongly CD10-positive, CD34+, TdT+, CLA-, CD20- cells suggests that these cells are more immature than the larger population of weakly CD10-positive, CD34-, TdT-, CLA+, CD20+ cells. We studied cells from 25 patients with CD10-positive ALL to determine if they demonstrate the same phenotypic heterogeneity as found in cBML.

Comparison of cALL cells that were strongly v weakly CD10 positive revealed differences in expression of differentiation antigens CD34, C μ , CLA, and PNA that were similar to those observed in cBML. In contrast, CD20 and TdT expression were not related to CD10 intensity of the cALL cells as they are in normal marrow. Indeed, CD20 expression was significantly higher in strongly CD10-positive cALL than in weakly CD10-positive cALL, opposite to the difference found in normal marrow. In contrast to cBML, cALL cells retain the same quantitative TdT expression despite the presence of CD20, CLA, or C μ . In normal marrow, the loss of TdT with acquisition of CD20, CLA, or C μ is nearly complete. C μ expression in cALL was negatively correlated with CD34 expression and positively correlated with CLA expression, as in cBML. However, in contrast to cBML, TdT and CD20 expression were unrelated to the presence of C μ in cALL. This is particularly striking in the case of TdT/C μ coexpression, which is common in cALL but exceedingly rare in cBML. TdT+/C μ + cells occurred with a frequency

Table 2. Characteristics of Sorted cALL Subpopulations

	CD20+	CD20-	P*	CD34+	CD34-	P	CD10+	CD10-	P
CD10 density (MFI)	62	62	NS	64	51	<.05	—	—	—
FALS†	785	720	<.02	841	774	<.01	860	818	NS
TdT intensity (fg/cell)	3.1	3.9	<.05	5.4	4.9	NS	3.2	3.1	NS
Cytoplasmic Ig (%)	58.4	58.1	NS	14.0	39.7	<.05	29.0	37.6	NS
S + G2 + M (%)	19.1	20.7	NS	13.7	13.3	NS	22.7	23.8	NS

*P values based on paired t test.

†Relative light scatter intensity.

NS, not significant.

of 1/4,000 marrow CD10-positive cells. Previous studies have also demonstrated the rarity of the TdT+/Cμ+ phenotype.²³

A potential complicating factor in investigations of cell lineage and differentiation using malignant cells from different patients is unrecognized heterogeneity in these patients with regard to causation or natural history that might obscure differentiation related differences. To address this issue, we studied subpopulations of cells from individual patients that were positive or negative for CD34, CD20, or CD10. The findings substantially corroborated the data obtained from study of separate patients. CD34 expression in cALL subpopulations was correlated with CD10 intensity and inversely related to Cμ expression, as is the case in cBML. CD20 expression in cALL subpopulations was unrelated to CD10 or Cμ expression, in contrast to cBMLs. TdT intensity in cALL subpopulations was unrelated to CD34 expression but inversely related to CD20 expression. The latter finding is the only occasion when TdT or CD20 expression in cALL showed a correlation similar to that found in cBMLs. The differences described were not likely to be due to differences in cell cycle, since there was no difference in cell cycle distribution between sorted subpopulations of cells positive or negative for CD10, CD34, and CD20.

The evidence presented above suggests that both from patient to patient and within individual patients, cALL cells express the markers CD34, CLA, CD10, and Cμ in a coordinated fashion similar to cBMLs. CD34-positive cALL cells show increased expression of CD10 and reduced CLA and Cμ expression, suggesting that they may represent a phenotypically more "immature" form of cALL than CD34-negative cALL (Fig 5). Classification of differentiation stages of cALL on the basis of CD34 or CLA expression may therefore be more biologically relevant than classification of cALL on the basis of CD20 expression.⁸

The data presented in this study, however, indicate certain phenotypic differences in cALL cells as compared with the bone marrow CD10-positive cells which are widely considered their normal counterparts.²⁴ There are several possible

explanations for this observation. The differences in TdT and CD20 expression between cBML and cALL may result from the loss of specific normal regulatory signals as a consequence of aberrant differentiation in cALL. The phenotypic differences may merely reflect a generalized disorganization of gene expression in malignant cells, but it is noteworthy in this regard that in cALL the expression of CD34, Cμ, CLA, and CD10 appears to be coordinated. Alternatively, the observed cALL phenotypes may reflect normal differentiation stages that are transient or found only in rare subpopulations of normal cells. Finally, modulation or down-regulation of certain cell markers may occur in cALL for reasons unrelated to cell differentiation.

The hypothesis of differentiation related subpopulations of cBML is supported by the strong relationships demonstrated in normal marrow between expression of TdT or Cμ and CD10, CLA, CD34, and CD20 (Figs 3B and 4B). The strong inverse association among Cμ, CD20, CLA, and TdT expression in cBML suggests the possibility of inhibition of TdT production by one of these proteins. TdT has been shown to be mutagenic during in vitro DNA synthesis,²⁵ and is therefore potentially detrimental to a proliferating mature B cell. The biologic consequences of persistent TdT expression in proliferating ALL cells is at present unknown.

Identification of differentiation related subtypes of cALL may provide useful clinical information. Weakly CD10-positive, CD34+ cALL seems to be more prevalent in adults, but the patient sample in this study is too small to make any statement concerning prognosis. Prognosis in cALL has been correlated with CD10 intensity^{25a} and Cμ expression,²⁶ two features that appear to be related to each other in this study. Prognosis in ALL is not related to TdT activity.¹⁴ Comparison of antigen expression during in vitro differentiation of cALL and cBML cells under conditions that permit cell growth²⁷ will be important in determining the biologic significance of the phenotypic differences observed in this study.

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Serum Immunoreactive Erythropoietin in Children With Cyanotic and Acyanotic Congenital Heart Disease

By Per Hågå, P. Mary Cotes, Janice A. Till, Barbara D. Minty, and Elliot A. Shinebourne

Serum immunoreactive erythropoietin (siEp) was measured in 27 cyanotic and 21 acyanotic children with congenital heart disease, age 4 months to 10 years. The geometric mean value was 9 mIU/mL for each group with 95% range from 3 to 26 mIU/mL and 4 to 22 mIU/mL for the cyanotic and acyanotic subjects, respectively. The levels are similar to those found in normal adults using the same assay system. Three cyanotic subjects showed increased siEp values. One was anemic relative to his hypoxemia, and the other two showed signs of increasing hypoxia. There was a significant negative correlation between siEp and arterial oxygen content. However, siEp

did not correlate significantly with hemoglobin, hematocrit, PaO₂, or SaO₂. Despite normal siEp levels, the cyanotic children showed compensatory erythropoiesis with significantly elevated hemoglobin and hematocrit levels, which did correlate inversely with PaO₂ and SaO₂. Arterial oxygen content was also significantly higher in the cyanotic subjects ($p < 0.02$). The cyanotic children seemed to display the same pattern as observed in man and animals exposed to prolonged hypobaric hypoxia, where after an initial rise in erythropoietin values the levels fall to normal, while increased erythropoiesis is sustained.

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THE HYPOXEMIA of cyanotic congenital heart disease (CCHD) causes erythrocytosis, with a concomitant increase in blood volume.^{1,2} The erythrocytosis is classified as a secondary appropriate polycythemia because the increased red cell mass is a physiologic response to the inadequate tissue oxygenation.¹

With increasing hematocrit the viscosity of blood rises, and this in turn may impair oxygen delivery to the tissues. The increased blood volume appears partly to counteract the increase in viscosity by lowering peripheral vascular resistance; there has been much debate however, on what is the optimal hemoglobin/hematocrit value for oxygen transport.³ In some patients with CCHD the erythrocytosis appears to be excessive, and they seem to benefit from reducing the hematocrit through partial exchange transfusion with plasma or 5% albumin.^{4,5}

Traditionally the secondary polycythemias are considered to have increased erythropoietin (Ep) levels in serum or urine, in contrast to the primary polycythemias, which are epitomized by polycythemia rubra vera.^{1,3,6} In recent investigations employing radioimmunoassay, this distinction is generally upheld⁷⁻⁹; however, some studies have found that serum immunoreactive erythropoietin (siEp) levels in secondary hypoxia-induced erythrocytosis may be either normal or increased.^{10,11} Although the concept of secondary polycythemia may be advantageous in terms of a diagnostic work-up, the lumping together of widely different disease states as a single entity clearly is unsatisfactory. The availability of

reliable radioimmunoassays for erythropoietin that can detect serum values in the normal range has made it opportune to study the different clinical conditions comprising secondary polycythemia individually.

Only one study specifically aimed at examining Ep levels in CCHD has been reported. This study employed a polycythemic mouse bioassay, and it was found that 13 out of 17 subjects had increased plasma concentrations, with 7 having very high values.¹² The adult patients with CCHD included in papers on polycythemia have shown increased levels.^{7,11,13} Apart from studies at birth and in the first few weeks of life,¹⁴⁻¹⁶ the literature contains little or no data on Ep levels in children. We report here on siEp concentrations in 48 children with congenital heart disease.

MATERIALS AND METHODS

The subjects of the study were patients undergoing cardiac catheterization or about to have cardiac surgery. The catheterized patients were under general anesthesia with a FiO₂ of 33%, in accordance with hospital routine. Blood was sampled from the catheter at the start of the procedure. In the patients about to be operated on, blood was obtained from an arterial line after the patient was stabilized under general anesthesia.

Twenty-seven cyanotic children and twenty-one acyanotic children having congenital heart disease were studied. Their age ranged from 4 months to 10 years. Cyanosis or arterial desaturation was defined as a systemic arterial oxygen saturation (SaO₂) of less than 94%. Clinical findings are shown in Table 1. Growth was assessed on the standard height and weight charts of Tanner & Whitehouse (Castlemead Publications, UK). None of the patients showed signs of heart failure at the time of study. Four of the cyanotic and five of the acyanotic children were receiving antifailure treatment.

Only infants more than 4 months of age were studied so that measurements would not be influenced by hematological changes occurring at birth. Similarly, the nadir of the physiological anemia of infancy was avoided. Children who were preterm and/or small for gestational age were included only if they were older than one year of age at the time of the study. None of the subjects had evidence of renal disease, and all had normal serum levels of creatinine and urea.

Hematological indices were determined using a Coulter Counter Model S 880 (Coulter Electronics). Serum ferritin was determined by radioimmunoassay (Amersham International, Chalfont, England); values lower than 16 µg/L signify iron deficiency in this assay. Blood gas measurements were performed on a Corning 178 pH/Blood Gas Analyzer (Corning Medical), and arterial oxygen

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Table 1. Clinical Data on the Subjects of the Study

	Cyanotics	Acyanotics
Age (yr)*	2.7 (4/12-10.0) n = 27	3.2 (4/12-8.5) n = 21
Length (centiles)†	15 th (0.0-95 th) n = 27	42 nd (0.0-99 th) n = 21
Weight (centiles)†	6 th (0.0-76 th) n = 27	19 th (0.0-98 th) n = 21
Diagnosis	TOF ± shunt (10) PA ± VSD ± shunt (4) TGA ± VSD (4) Double inlet ventricles (3) Double outlet ventricles (2) TA + shunt (2) TAPVC (1) Very complex (1)	PS (5) VSD ± PS (4) TOF (3) Coarctation (2) ASD (1) Corr. transpos. (1) PA + VSD + shunt (1) PDA (1) TA + Kreuzer (1) TGA + Mustard (1) TOF + correction (1)

Number of patients in parenthesis.

*Values are means (range).

†Values are means (range). $p > 0.05$ for cyanotic vs acyanotic group (two-tailed unpaired t-test, degrees of freedom = 44). Centiles refer to those of normal children (Tanner & Whitehouse, Castlemead Publications 1983).

Abbreviations: ASD, atrial septum defect; PA, pulmonary atresia; PDA, patent ductus arteriosus; PS, pulmonary stenosis; TA, tricuspid atresia; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect; shunt, modified Blalock-Taussig aortopulmonary shunt; TAPVC, total anomalous pulmonary venous connections.

saturation was determined spectrophotometrically in a IL 282 CoOximeter (Instrumentation Laboratory). Standard P_{50} (pH 7.40, 37°C) was measured as described by Holter et al.¹⁷ Blood from five normal children, age 19 months to 8 years, had levels of 3.3 ± 0.3 kPa* (mean $\pm$ SD), using this method.

SiEp was estimated by a modification of the radioimmunoassay method described and validated by the method of Egrie.¹⁸ ¹²⁵I recombinant human erythropoietin (Amersham International, IM 178) was used as tracer antigen, and the assay diluent was 0.04 M phosphate, 0.01 M disodium EDTA (pH 7.5) containing 0.15 M NaCl, 0.01% sodium azide, and human plasmaprotein fraction (2 mL/100 mL) (Lister, Elstree; 4.5 g protein/100 mL). The WHO second international reference preparation of erythropoietin (2nd IRP) was used as standard.¹⁹ Serum samples from blood allowed to clot at room temperature were stored at -20°C until analysis. Each serum sample was tested in two assay tubes, usually at two-dose levels, but 12 sera were tested as two identical replicates. All samples were examined as a batch in a single assay to exclude interassay variability. Forty-nine samples tested in the assay at two or more dose levels gave consistent potency estimates at all dose levels, indicating parallelism of dose dilution curves of sera and the second IRP. The between assay coefficient of variation of estimates was 11%.

Statistical difference was tested by unpaired student's t-test; correlation was tested by linear regression. SiEp shows a log normal

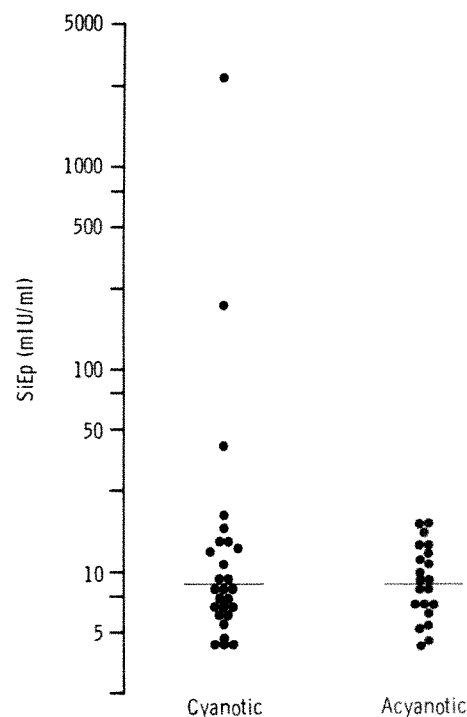


Fig 1. Serum immunoreactive erythropoietin levels in 27 cyanotic and 21 acyanotic children with congenital heart disease. The three highest values are those of the subjects in Table 3. Horizontal lines depict the geometric mean values for the two groups (the two highest values in the cyanotic group were omitted in the statistical evaluation. See Materials and Methods).

distribution and was therefore tested after logarithmic transformation; means were expressed as geometric means. Some of the acyanotic patients displayed hyperoxemia on being artificially ventilated at a FiO_2 of 33% and PO_2 values above 15 kPa were excluded from analysis. Two cyanotic patients (subjects II & III in Table 3) had SiEp values 6.0 and 10.8 standard deviations, respectively, above the rest of the group. For this reason they were excluded from the statistical analysis. The study was approved by the Brompton Hospital Ethical Committee.

RESULTS

The estimates of SiEp are shown in Fig 1 and Table 2. The geometric mean was 9 mIU/mL for each group, with a 95% range† from 3 to 26 mIU/mL for cyanotic and 4 to 22 mIU/mL for acyanotic subjects. The difference is not statistically significant. The levels are similar to those of 21 normal adults using the same assay system (geometric mean 8 mIU/mL, 95% range from 4 to 16 mIU/mL).

Three cyanotic patients (subjects I, II, III; Table 3) had SiEp values 3.8, 7.6, and 13.5 standard deviations, respectively, higher than their counterparts (Fig 1). Subject I, with arterial oxygen saturation 80.9% and hemoglobin concentration 12.7 g/dL, may be considered anemic in relation to his hypoxemia. This relative anemia was most likely due to iron deficiency (serum ferritin 8 μ g/L). This may explain the

* (1 kPa = 7.5 mm Hg).

†The range within which 95% of the observations are predicted to fall.

Table 2. Hematologic and Physiologic Data of the Study Groups

	n	Cyanotics	n	Acyanotics
Erythropoietin (mIU/mL)	25	9 (3-26)	21	9 (4-22)
Hemoglobin (g/dL)	25	15.1 ± 1.8*	21	11.7 ± 1.4
Hematocrit	25	0.46 ± 0.05*	21	0.35 ± 0.04
PO ₂ (kPa)†	25	7.2 ± 1.2	17	>9.9
SaO ₂ (%)	25	84.0 ± 7.8	19	97.5 ± 1.2
Arterial O ₂ content (mL/dL)	25	17.1 ± 2.2‡	19	15.5 ± 1.8
P ₅₀ (kPa)†	9	3.9 ± 0.4‡	4	3.5 ± 0.2

Values are means ± SD, except for erythropoietin values, which are geometric means (95% range).

* $P < 0.001$ when compared with the values of acyanotics (two-tailed unpaired t -test, degrees of freedom = 44).

†1 kPa = 7.5 mm Hg; 1 mm Hg = 0.133 kPa.

‡ $P < 0.02$ when compared with the values of acyanotics (two-tailed unpaired t -test, degrees of freedom = 42).

raised siEp concentration. Subjects II and III had both shown symptoms and signs of increasing cyanosis and hypoxia during the months leading up to hospital admission. In addition, subject III suffered from iron deficiency anemia, as judged from red cell indices and serum ferritin (Table 3).

Hemoglobin concentration and hematocrit were significantly higher in the cyanotic than the acyanotic subjects ($p < 0.001$, $n = 46$). Arterial oxygen content ($1.36 \times \text{hb} \times \text{SaO}_2$) was also significantly higher in the cyanotic group ($p < 0.02$, $n = 44$) (Table 2).

The siEp concentrations did not show any significant correlation with hemoglobin concentration or hematocrit, or with systemic arterial oxygen tension (PaO₂) or SaO₂. This was so both for the cyanotic and acyanotic patients as well as for the two groups combined. However, there was a significant negative correlation between siEp and arterial oxygen content in the cyanotic group ($r = -0.56$, $P < 0.01$, $n = 25$) (Fig 2). The inverse correlation was also significant for all patients combined, although to a lesser extent ($r = -0.43$, $P < 0.01$, $n = 44$).

Hematocrit was inversely correlated with arterial PO₂ and oxygen saturation for both the cyanotic group and all subjects combined ($p < 0.05$ in all instances). Hemoglobin concentration showed the same pattern, but the relation did not attain significance in the cyanotic group. The best correlation was obtained between hematocrit and SaO₂ in the total sample ($r = -0.74$, $P < 0.001$, $n = 44$).

Table 3. Data on the Three Subjects Showing Increased siEp Levels

Subjects	I	II	III
Age (yrs)	1½	½	1½
Cardiac diagnosis	TGA + VSD	TGA	PA + VSD + shunt
Erythropoietin (mIU/mL)	42	211	2695
Hemoglobin (g/dL)	12.7	15.0	16.2
Hematocrit	0.41	0.47	0.54
MCV (fl)*	79	86	64
MCH (pg)*	24.5	27.6	19.1
Ferritin (g/L)	8	24	6
PO ₂ (kPa)	7.5	5.0	5.9
SaO ₂ (%)	80.9	63.9	64.0

*Normal values v age (see Dacie JV, Lewis SM [eds]: Practical Haematology. London, Churchill Livingstone, 1984, p 8).

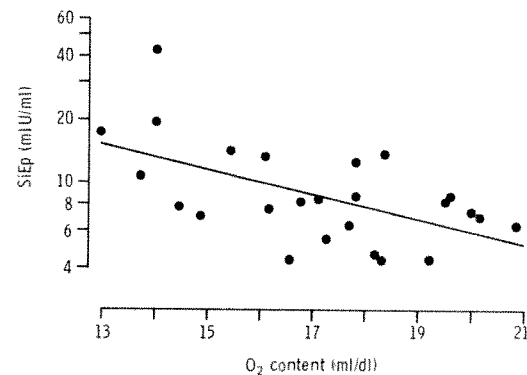


Fig 2. Serum immunoreactive erythropoietin levels vs oxygen content ($1.36 \times \text{Hb} \times \text{SaO}_2$) of the cyanotic subjects. The correlation is statistically significant ($r = -0.56$, $P < 0.01$, $n = 25$).

Standard P₅₀ was measured in arterial blood from 13 patients. In cyanotic subjects ($n = 9$), the mean P₅₀ was 3.9 kPa (range 3.3-4.4 kPa), which was significantly higher than in normal children ($P < 0.02$, $n = 12$). No correlation with siEp values was noted. There was a positive correlation between P₅₀ and hemoglobin and hematocrit levels that did not attain significance. However, when both cyanotic ($n = 9$) and acyanotic ($n = 4$) patients were combined, the relations became significant ($r = 0.71$, $P < 0.01$ vs hemoglobin, and $r = 0.72$, $P < 0.01$, vs hematocrit).

From three cyanotic patients who had aortopulmonary shunt operations, serial samples were collected for siEp assay in conjunction with blood gas sampling. For each patient, PaO₂ did not improve during the study period, and siEp levels remained unchanged (Fig 3). In another patient, age 3 years, blood was sampled on two occasions, 5 months apart. During this period, her condition remained stable, and estimates of siEp were 7 mIU/mL and 11 mIU/mL on the two occasions, with PaO₂ of 8.3 and 8.6 kPa, respectively.

DISCUSSION

The low siEp concentrations found in the cyanotic group were unexpected. Our findings are not in agreement with either the general notion of raised Ep levels in secondary

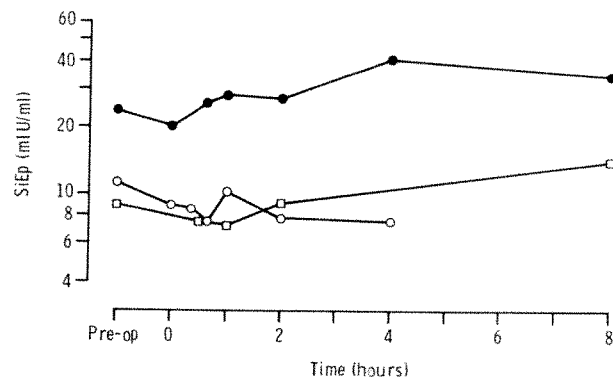


Fig 3. Serum immunoreactive erythropoietin levels of three subjects taken at the start, during, and after operation for insertion of modified Blalock-Taussig aortopulmonary shunt. The arterial PO₂ remained unchanged during the study period.

polycythemia nor with the raised levels reported in adults with CCHD in other radioimmunoassay studies. In fact, Koeffler and Goldwasser found the patients with cyanotic heart disease to have the highest average concentration among the secondary polycythemia subjects.⁷ Our findings cannot be explained by insufficient severity of hypoxemia, since the PaO₂ ranged from 5.0 to 8.9 kPa, with an even spread of values.

A FiO₂ of 33% is used in general anesthesia to counteract the negative effects of artificial ventilation on oxygenation. Some acyanotic subjects displayed hyperoxemia when ventilated in this way, and there is a possibility that the SaO₂ of some of the cyanotic patients with a high pulmonary blood flow was also increased by artificial ventilation.

Oxygen delivery to the tissues depends on many factors, in particular on the total number of circulating erythrocytes, PaO₂, SaO₂, the position of the oxygen-hemoglobin dissociation curve, cardiac output, and regional blood flow.

A possible reason for these low siEp values in children with CCHD is that their hypoxemia was totally compensated, and consequently no tissue hypoxia was experienced. Compensatory mechanisms to counteract tissue hypoxia clearly operated in the patients of our study group. We did not measure red cell mass, but hemoglobin levels and hematocrit were well above normal in our cyanotic patients and, moreover, were inversely correlated with PO₂ and SaO₂, indicating increasing production according to increasing needs. A rightward shift of the oxygen-hemoglobin dissociation curve is considered advantageous for oxygen delivery when lowered PaO₂ is caused by right to left shunting of blood,²⁰ which is in line with experimental findings.²¹ We found an increase in standard P₅₀ in the cyanotic patients tested in our study (Table 2). Furthermore, for all subjects tested, P₅₀ levels correlated significantly with hemoglobin concentrations and hematocrit. Most importantly, the arterial oxygen content was found to be significantly higher in the cyanotic than in the acyanotic subjects (Table 2). However, although we have shown compensatory adjustments at work, whether total compensation of hypoxemia occurs is not answered by this investigation. Total compensation for any deficiency state in a child would entail "normal" growth and development. Our patients showed reduced growth, both in height and weight, as is a common finding in CCHD. However, the growth impairment was not significantly more severe in the cyanotic than the acyanotic group. Hypoxia seems to be only one of several factors, albeit an important one, responsible for impaired growth in congenital heart disease.²² A recent study found an inverse association between cognitive function and time of corrective surgery (6 months to 6 years) in children with simple transposition, suggesting that the length of hypoxemic exposure has an adverse effect and that the hypoxemia is not totally compensated.²³ On balance, we believe that total correction of tissue hypoxia most likely is not achieved and that the low Ep values cannot be fully explained through such a mechanism. One may argue that through reduction in growth, oxygen consumption, and physical exercise, oxygen needs are reduced to a lower level whereby equilibrium is reestablished through lower demands. However, such a concept seems unlikely in infants

and children because of the major emphasis during this period on growth and development.

When exposed to hypobaric hypoxia, both experimental animals and humans show an initial rise in erythropoietin concentration that falls gradually to normal levels in the ensuing days or weeks. Even though the Ep levels return to normal, increased erythropoiesis is sustained, with a continuous rise in hemoglobin concentrations and hematocrit levels.²⁴⁻²⁷ Looking at other subjects chronically exposed to hypoxia, we find little data on erythropoietin secretion in persons living permanently at high altitude. However, in one study, urinary Ep was increased in only two out of six subjects.²⁸ In patients with severe hypoxic lung disease with an associated polycythemia, siEp was above normal in only 5 out of 10 patients.¹⁰ Thus, from both experimental work in animals and humans as well as clinical studies, the axiom of a direct relationship between the degree of hypoxemia and serum or urinary erythropoietin concentrations is not borne out under long-term hypoxic conditions. We believe the cyanotic patients in the present study fit the pattern outlined above and that their low siEp levels may be explained in the context of chronic hypobaric hypoxia.

The raised siEp concentrations of the three patients in Table 3 must be explained differently. Subject I was relative to his cyanotic heart disease anemic (SaO₂ 80.9%) at the time of study, most likely due to iron deficiency, and this may be the reason for his elevated siEp value. The other two subjects had both shown severe signs and symptoms of increasing cyanosis and hypoxia during the preceding months. They were thus not in a "stable chronic state," and both in humans and animals exposed to prolonged hypoxic conditions, it has been shown that increasing the hypoxic stimulus causes a renewed rise in Ep levels.^{24,26,27}

Our finding of a significant negative correlation between siEp levels and arterial oxygen content in CCHD (Fig 2) indicates that oxygen supply is a factor in the regulation of erythropoietin production even when the Ep concentrations are in the "normal" range.

Single determinations of concentrations of erythropoietin both as a measure of the production rate of the hormone and as a reflection of the serum levels over time may be seen as wanting. Diurnal variations in siEp values in humans,^{29,30} as well as fluctuating levels in a polycythemic adult, have been observed.¹¹ Fig 3 shows that multiple samples from the same individual showed fairly stable levels of siEp. This indicates that the single measurements of the patients in our study can reasonably be expected to give good estimates of the steady state siEp levels.

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Effect of Calcium Ion Concentration on the Ability of Fibrinogen and von Willebrand Factor to Support the ADP-induced Aggregation of Human Platelets

By E.J. Harfenist, M.A. Packham, R.L. Kiriough-Rathbone, M. Cattaneo, and J.F. Mustard

To investigate the suggestion that von Willebrand factor (vWf) can substitute for fibrinogen in supporting ADP-induced aggregation of human platelets, we studied platelet reactions in two media: (1) a high calcium medium, Tyrode-albumin solution containing calcium ions in the physiological range of 2 mmol/L, and (2) a low calcium medium, modified Tyrode-albumin solution from which calcium salt was omitted (calcium ion concentration approximately 20 μ mol/L). In the high calcium medium vWf even at concentrations up to six times as high as physiological, showed little or no potentiation of ADP-induced platelet aggregation, whereas fibrinogen strongly potentiated reversible aggregation without thromboxane formation or release of granule contents. In the low calcium medium, either vWf or fibrinogen supported biphasic aggregation in

response to ADP, with thromboxane formation and release of granule contents. Aspirin and the thromboxane receptor blocker BM 13.177 inhibited these secondary responses to von Willebrand factor, indicating that they require thromboxane A_2 formation and feedback amplification by thromboxane A_2 . A monoclonal antibody, 10E5, to the platelet glycoprotein IIb/IIIa complex inhibited both primary and secondary aggregation. Although vWf supports ADP-induced aggregation when the concentration of ionized calcium is in the micromolar range, it does not support ADP-induced aggregation in the presence of a concentration of ionized calcium in the physiological range, indicating that vWf probably cannot substitute for fibrinogen in supporting ADP-induced aggregation *in vivo*.

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IT IS WELL KNOWN THAT von Willebrand factor (vWf), by binding to glycoprotein Ib on the platelet membrane, mediates the adherence of platelets to the subendothelium of an injured vessel wall.¹ In addition, it is generally accepted that fibrinogen supports ADP-induced aggregation of human platelets and in doing so binds to the glycoprotein IIb/IIIa complex on the platelet membrane.²⁻⁷ In the absence of fibrinogen, vWf also binds to this complex when platelets are stimulated with ADP⁸⁻¹⁵ and has been reported to support the ADP-induced aggregation of washed platelets resuspended in a balanced salt solution to which no calcium salt has been added.⁸ Addition of vWf to citrated platelet-rich plasma (PRP) from afibrinogenemic patients has also been observed to cause aggregation in response to ADP.¹⁶

We¹⁷ and others¹⁸ have observed differences in the responses of human platelets to ADP in PRP, depending on whether hirudin or citrate has been used as the anticoagulant, and have ascribed these observations to differences in the concentration of ionized calcium (Ca^{2+}), since the responses can be reproduced in artificial media by using different concentrations of Ca^{2+} .^{17,19,20} In a medium containing a physiological Ca^{2+} concentration (1 to 2 mmol/L), ADP in the presence of fibrinogen induces only one phase of platelet aggregation, which is reversible and does not involve thromboxane formation or the release of granule contents, regardless of the concentration of ADP or fibrinogen. This is similar to the response to ADP in PRP anticoagulated with hirudin where the concentration of Ca^{2+} is 1.1 mmol/L.¹⁸ In contrast, in a balanced salt medium without added Ca^{2+} , ADP in the presence of fibrinogen induces two phases of aggregation (essentially irreversible), thromboxane formation and the release of granule contents.^{17,19,20} This is similar to the response to ADP in citrated PRP in which the concentration of Ca^{2+} is approximately 40 μ mol/L.¹⁸ The effect of vWf on ADP-induced aggregation appears not to have been tested in media containing physiological concentrations of Ca^{2+} , and the present experiments were designed to compare fibrinogen and vWf with respect to their effects

on the aggregation responses of human platelets to ADP in media with different concentrations of Ca^{2+} .

MATERIALS AND METHODS

Materials. ADP, aspirin, and ristocetin were from Sigma Chemical Co, St Louis. Imipramine was from Geigy Canada Ltd, Montreal, Quebec. The thromboxane receptor blocker BM 13.177, (2-[benzene-sulfonamido]-ethyl)-phenoxyacetic acid, was from Boehringer Mannheim, West Germany. F(ab')₂ fragments of anti-human fibrinogen (goat), and nonimmune F(ab')₂ fragments (goat) were from CooperBiomedical, Inc (Cappel), Malvern, PA. Mouse monoclonal antibody (10E5) to the glycoprotein IIb/IIIa complex was kindly supplied by Dr Barry Collier, Stony Brook, NY. ¹⁴C-Serotonin (5-hydroxytryptamine-3'-¹⁴C-creatinine sulfate) was from Amersham Corp, Arlington Heights, IL. The radioimmunoassay kit for thromboxane B₂ (TXB₂) was supplied by New England Nuclear Canada, Lachine, Quebec, and that for fibrinopeptide A was an

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RIA-Quant FPA Test Kit from Mallinckrodt, Inc, St Louis. Bovine albumin was Pentex fraction V from Miles Laboratories, Elkhart, IN. Human fibrinogen (Grade L) was from AB Kabi, Stockholm. Lyophilized platelets were from Bio/Data Corp, Hatboro, PA. Apyrase was prepared from potatoes by the method of Molnar and Lorand.²¹

Preparation of vWf. vWf was prepared from factor VIII concentrates (generously provided by Po Wah, Connaught Laboratories, Downsview, Ontario) by adsorption to bentonite and polyethylene glycol precipitations followed by chromatography on Bio-Gel A-15m, as described by McKee and colleagues.^{22,23} Characterization by gel electrophoresis in the presence of sodium dodecyl sulfate, using 4% acrylamide (reduced samples) or 1% agarose (nonreduced samples) indicated a monomer with a molecular weight of approximately 2.1×10^5 existing as multimers ranging in size from 2 to 3×10^6 to 8 to 10×10^6 , similar to those in normal plasma.²⁴ Ristocetin cofactor activities were determined using formaldehyde-treated platelets²⁵ or lyophilized platelets; activities were in the range of 24 to 100 U/mg. The vWf, dissolved in phosphate-buffered saline, pH 7.4, was stored in small aliquots at -70°C , and samples were centrifuged for 2 minutes at $12,000 \times g$ (Eppendorf centrifuge, Brinkmann, Rexdale, Ontario) to remove aggregates immediately before use.

Preparation of citrated PRP from afibrinogenemic patient. Citrated PRP was prepared from blood anticoagulated with 3.8% trisodium citrate, one part to nine parts blood. The platelet count was $4 \times 10^5/\mu\text{L}$.

Preparation of platelet suspensions. Suspensions of washed platelets from human blood were prepared as described previously.^{19,26} Two final suspending media were used; both were essentially Tyrode solutions containing 0.35% albumin and apyrase (Tyrode-albumin). The first medium (hereafter referred to as the high Ca^{2+} medium) contained approximately physiological concentrations of divalent cations: 2 mmol/L Ca^{2+} and 1 mmol/L Mg^{2+} ; the second medium (hereafter referred to as the low Ca^{2+} medium) was a modified Tyrode-albumin solution from which Ca^{2+} was omitted; the Ca^{2+} concentration was approximately 20 $\mu\text{mol/L}$ as measured by atomic absorption spectrometry. The platelets were prelabeled with ^{14}C -serotonin (0.2 $\mu\text{Ci/mL}$ of platelet suspension) in the first washing fluid to permit the measurement of the release of granule contents, as described previously.²⁷ Imipramine at a concentration of 5 $\mu\text{mol/L}$ was added at the time of ADP addition to prevent reuptake of released serotonin.

Platelet aggregation. Aggregation was studied by recording light transmission through 1-mL samples of platelet suspension ($5 \times 10^5/\mu\text{L}$) stirred at 1100 rpm at 37°C in a cuvette in an aggregation

module (Payton Associates, Scarborough, Ontario). For measurement of TXB_2 formation and release of granule contents, reactions were stopped by centrifugation at $12,000 \times g$ for 1 minute; the supernatant solutions were removed immediately, and aliquots were used for ^{14}C determination or stored at -70°C until assayed for TXB_2 by radioimmunoassay. Concentrations of all materials added to platelet suspensions are given as final concentrations after all additions. In a few experiments, platelets were fixed by the addition of 1% formaldehyde²⁸ and examined microscopically.

Patients with vWf deficiency. Two patients with von Willebrand disease subtype III, with nondetectable or minute amounts of vWf in plasma and in platelets, were B.B. and B.Z., described previously.²⁹

Patient with afibrinogenemia. The afibrinogenemic patient had plasma and platelet fibrinogen concentrations (measured by means of a fibrinopeptide A radioimmunoassay) of 3 $\mu\text{g/mL}$ and 8.6 $\mu\text{g}/10^9$ platelets, respectively.³⁰

RESULTS

Comparison of effects of fibrinogen and vWf on ADP-induced platelet aggregation in the high Ca^{2+} medium (2 mmol/L). Addition of ADP (1 to 20 $\mu\text{mol/L}$) to suspensions of washed platelets containing 2 mmol/L Ca^{2+} caused a weak, reversible aggregation response (Fig 1). The extent of the change in light transmission was proportional to the concentration of ADP, and microscopic examination of formaldehyde-fixed platelets showed small aggregates at the peak of the response. That this response was not caused by traces of fibrinogen or vWf was indicated by the lack of inhibition by F(ab')_2 fragments of antifibrinogen (225 $\mu\text{g/mL}$) and by the fact that a similar weak response was observed with platelets from two patients with a severe deficiency of vWf, a response also not inhibited by F(ab')_2 fragments of antifibrinogen. (These F(ab')_2 fragments did inhibit potentiation of this response by fibrinogen, whereas nonimmune F(ab')_2 had no effect.³⁰) In this high Ca^{2+} medium, the addition of fibrinogen potentiated the ADP-induced aggregation of human platelets, but only the primary phase occurred, and it was followed by deaggregation (Fig 1A). Previously it has been shown that when only the primary phase of ADP-induced aggregation occurs upon the addition of fibrinogen, there is no appreciable formation of thromboxane or release of granule contents.^{17,20} In contrast, in seven out of nine experiments, the addition of vWf, in concentrations ranging from 30 to 90 $\mu\text{g/mL}$, had no effect or caused barely detectable potentiation of the aggregation induced by ADP alone; slight potentiation was observed in two experiments (Fig 1B). In none of these experiments was there appreciable TXB_2 formation or release of granule contents.

Comparison of effects of fibrinogen and vWf on ADP-induced platelet aggregation in the low Ca^{2+} medium (20 $\mu\text{mol/L}$). In the low Ca^{2+} medium, addition of ADP to suspensions of washed platelets caused a weak, reversible aggregation response similar to that observed in the presence of 2 mmol/L Ca^{2+} (Fig 2A). As shown in previous studies,^{17,20} the effect of added fibrinogen on the response of platelets to ADP depended on the concentration of Ca^{2+} in the suspending medium. Thus, in the low Ca^{2+} medium, fibrinogen-potentiated aggregation occurred in two phases, a primary, reversible phase followed by a secondary irreversible

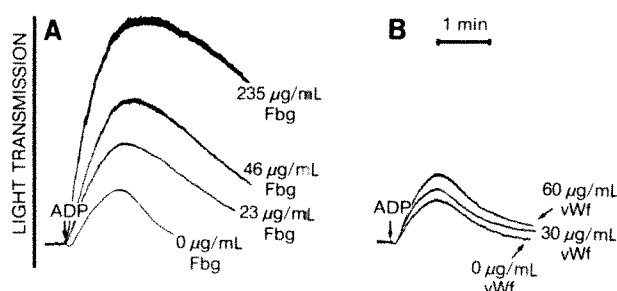


Fig 1. Responses of washed human platelets to ADP (10 $\mu\text{mol/L}$) in the high Ca^{2+} medium (Tyrode-albumin containing 2 mmol/L Ca^{2+}). (A) Fibrinogen (Fbg) added before ADP; typical of six experiments. (B) vWf added before ADP; representative of maximum potentiation, observed in only two out of nine experiments; in the other seven experiments vWf had no effect or caused a barely detectable potentiation of aggregation.

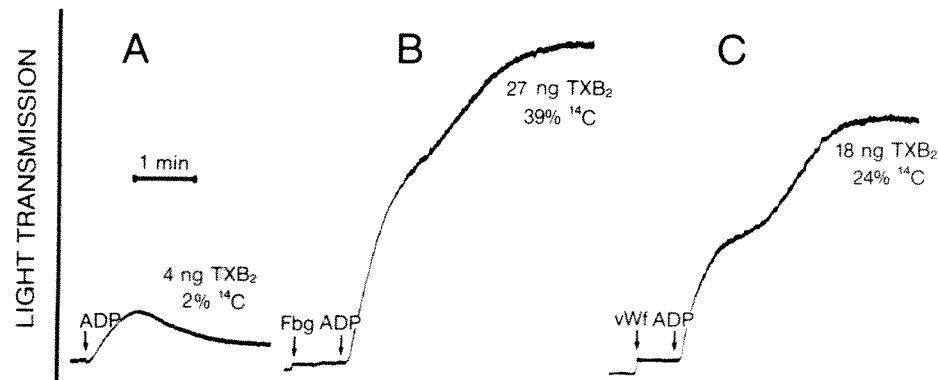


Fig 2. Responses of washed human platelets to ADP ($10 \mu\text{mol/L}$) in the low Ca^{2+} medium (modified Tyrode-albumin from which Ca^{2+} was omitted). (A) No additions. (B) Fibrinogen (Fbg, $64 \mu\text{g/mL}$) added before ADP. (C) vWf ($60 \mu\text{g/mL}$) added before ADP. Formation of TXB_2 ($\text{ng}/10^9$ platelets) and release of ^{14}C -serotonin (%) are shown beside the aggregation curves. Similar results were obtained in six experiments with fibrinogen at $11\text{--}400 \mu\text{g/mL}$ and vWf at $24\text{--}98 \mu\text{g/mL}$.

ible phase associated with the formation of TXB_2 and the release of ^{14}C -serotonin (Fig 2B). In the low Ca^{2+} medium, the effect of vWf on the response of platelets to ADP was similar to that caused by fibrinogen; vWf, at concentrations (30 to $90 \mu\text{g/mL}$) higher than the normal plasma concentration, potentiated ADP-induced aggregation, causing a biphasic response with the formation of TXB_2 and the release of ^{14}C -serotonin (Fig 2C). F(ab')_2 fragments of antifibrinogen had no effect on this potentiation, indicating that it was not caused by contaminating fibrinogen in the vWf preparation.

The potentiation of aggregation by vWf in the low Ca^{2+} medium as well as TXB_2 formation and the release of granule contents (Fig 3B) was inhibited by the addition of 1 or 2 mmol/L Ca^{2+} , aspirin, or the thromboxane receptor blocker BM 13.177 before ADP (Fig 3C, D, E). Aggregation was

abolished by the prior addition of 10E5, a monoclonal antibody to the glycoprotein IIb/IIIa complex (Fig 3F). This antibody not only prevented potentiation due to vWf, but also blocked the small aggregation response to ADP alone, although not the change in shape caused by ADP, as shown by the downward deflection of the aggregation recording, as previously observed by Collier and associates.³¹

In agreement with the observations of De Marco and colleagues,¹⁶ vWf ($50 \mu\text{g/mL}$) potentiated ADP-induced aggregation in citrated PRP from an afibrinogenemic patient.

DISCUSSION

The present results show that vWf, unlike fibrinogen, either fails to potentiate or causes only slight potentiation of ADP-induced aggregation in an artificial medium contain-

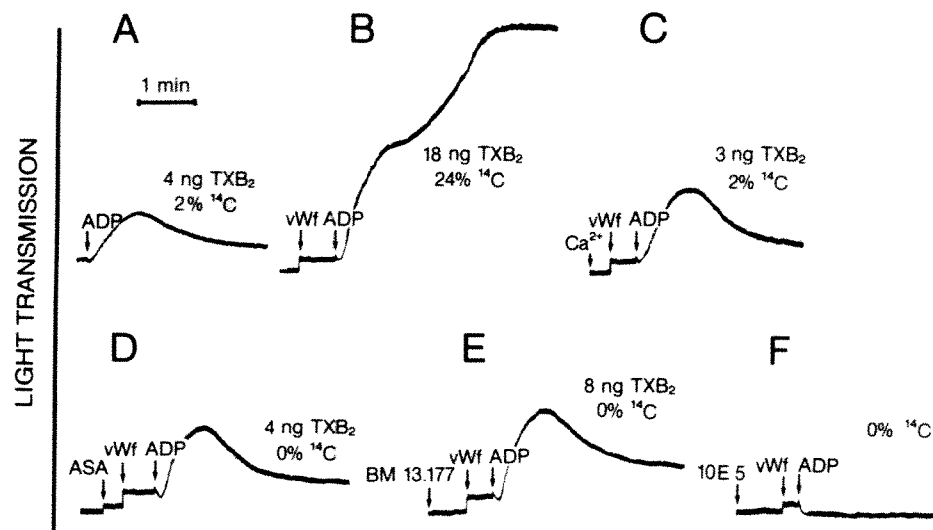


Fig 3. Inhibition of potentiation by vWf ($60 \mu\text{g/mL}$) of aggregation of washed human platelets stimulated by ADP ($10 \mu\text{mol/L}$) in the low Ca^{2+} medium. (A) No additions. (B) vWf only added. (C) Ca^{2+} (2 mmol/L) added before vWf and ADP. (D) Aspirin (ASA, $100 \mu\text{mol/L}$) added before vWf and ADP. (E) BM 13.177 ($100 \mu\text{mol/L}$) added before vWf and ADP. (F) 10E5 ($10 \mu\text{g/mL}$) added before vWf and ADP. Similar results were obtained for inhibition by Ca^{2+} , aspirin, or BM 13.177 when tested with vWf at $30\text{--}90 \mu\text{g/mL}$ (10E5 was tested with vWf at $30 \mu\text{g/mL}$). Formation of TXB_2 ($\text{ng}/10^9$ platelets) and release of ^{14}C -serotonin (%) are shown beside the aggregation curves. The aggregation curves from Figs 2A and 2C have been reproduced in Figs 3A and 3B, respectively, to facilitate estimations of inhibition.

ing a concentration of Ca^{2+} within the physiological range of 1 to 2 mmol/L. These experiments were done with concentrations of vWf ranging from 30 to 90 $\mu\text{g/mL}$, which are much higher than the normal plasma concentration of approximately 10 $\mu\text{g/mL}$. In contrast, as had been shown previously,^{2,26} fibrinogen strongly potentiates ADP-induced aggregation in the presence of physiological concentrations of Ca^{2+} , although aggregation is reversible regardless of the concentration of fibrinogen and does not involve thromboxane formation or release of granule contents. These observations lead one to question whether vWf plays a significant role in supporting ADP-induced platelet aggregation *in vivo*. It is possible, however, that *in vivo* there are other factors as yet not understood that enable this protein to contribute to the support of aggregation at physiological Ca^{2+} concentrations.

In accord with the findings of other investigators,^{8,16} vWf is similar to fibrinogen in its ability to potentiate ADP-induced platelet aggregation in media in which the concentration of Ca^{2+} is in the micromolar range; potentiation by either vWf or fibrinogen causes activation of the arachidonate pathway, leading to thromboxane formation, release of granule contents, and a biphasic aggregation response that is essentially irreversible. The low Ca^{2+} concentration in the plasma may have been responsible for the strong potentiation by vWf of ADP-induced aggregation in citrated PRP from the afibrinogenemic patient studied in the present investigation and from one of the afibrinogenemic patients (V.M.) studied by De Marco and colleagues.¹⁶ The inability of fibrinogen²⁰ or vWf to support second phase aggregation in the low Ca^{2+} medium in the presence of aspirin or the thromboxane receptor blocker BM 13.177³² confirms dependence on the arachidonate pathway that leads to thromboxane A_2 (TXA_2) formation. Inhibition by 10E5, the monoclonal antibody directed against the glycoprotein IIb/IIIa complex, indicates that the binding of vWf to this complex is

involved in potentiation of aggregation; 10E5 has previously been shown to inhibit fibrinogen support of ADP-induced aggregation and the binding of fibrinogen and vWf to activated platelets.^{31,33}

These experiments provide another example of the observation that treatments that cause close platelet contact in a medium containing a micromolar concentration of Ca^{2+} activate the arachidonate pathway, leading to thromboxane formation, release of granule contents, and secondary aggregation.¹⁷⁻²⁰ This effect of micromolar concentrations of Ca^{2+} on platelet responsiveness has been shown for aggregation by ADP or epinephrine in the presence of fibrinogen, for polylysine-induced agglutination, and for aggregation of chymotrypsin-treated platelets by fibrinogen²⁰ as well as for aggregation by ADP in the presence of vWf in the present experiments. This response of platelets to close platelet-to-platelet contact in a low Ca^{2+} medium requires feedback amplification by TXA_2 , since it is prevented by BM 13.177,²⁰ an inhibitor that blocks the thromboxane receptor. Since none of these agonists causes the secondary phase of aggregation, thromboxane formation, or the release of granule contents in a medium containing a physiological concentration of Ca^{2+} , this effect of close contact must be caused, at least *in vitro*, by low concentrations of Ca^{2+} , and it must be considered in all experiments using citrated PRP or suspensions of washed platelets in artificial media without added Ca^{2+} . Although some investigators have described the effect of a physiological concentration of Ca^{2+} as inhibition of ADP-induced aggregation,³⁴ it is apparent that the truly abnormal situation is a Ca^{2+} concentration in the micromolar range.

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Selective Uptake of [³H]Arachidonic Acid Into the Dense Tubular System of Human Platelets

By Michael Laposata, Charles M. Krueger, and Jeffrey E. Saffitz

We have used quantitative electron microscopic autoradiography to characterize the subcellular distribution of arachidonoyl phospholipids following brief (5 minutes) exposure of unstimulated human platelets to [³H]arachidonic acid. Labeled arachidonate was taken up rapidly and incorporated into phospholipids. Phospholipid radioactivity was preserved and spatially fixed during tissue processing for electron microscopy. Analysis of autoradiographs showed that following a brief exposure to 750 nmol/L [³H]arachidonate, there is selective labeling of an internal membrane compartment composed of the dense tubular system and the open canalicular system. The plasma

membrane, platelet granules, and nonmembranous cytoplasm were not labeled. Since the open canalicular system is continuous with the plasma membrane and since phospholipids in continuous membranes are freely diffusible, our observations indicate that [³H]arachidonate was incorporated into phospholipids within the dense tubular system and not the open canalicular system. Thus, the dense tubular system, known to contain cyclooxygenase activity, incorporates arachidonate selectively following brief exposure to this fatty acid, presumably to concentrate it in proximity to enzymes for icosanoid synthesis.

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THE PRODUCTION of icosanoids, oxygenated metabolites of arachidonic acid, requires cellular mechanisms for uptake and release of arachidonate from phospholipids. It has been demonstrated recently that icosanoid production can be impaired despite normal cyclooxygenase activity, an abundant supply of cellular arachidonate, and release of arachidonate from phospholipids.¹ In this situation, compromised production of icosanoids is due to markedly decreased activity of arachidonoyl-CoA synthetase, an enzyme that appears to function as an icosanoid precursor fatty acid uptake system in cells. Similar results have been obtained using a mutant cell line devoid of arachidonoyl-CoA synthetase activity.^{2,3} Thus, the process of arachidonate incorporation into the cell, the earliest step in the pathway from arachidonate to icosanoids, has an effect on the production of icosanoids following stimulation by agonists.

In this study, we investigated the incorporation of a five-minute pulse of 750 nmol/L [³H]arachidonate into subcellular membrane compartments in the platelet. Arachidonate metabolism and icosanoid production have been characterized extensively in blood platelets.⁴ Platelets contain a high affinity arachidonoyl-CoA synthetase, which permits selective accumulation of icosanoid precursor fatty acids that are rapidly esterified into phospholipids.^{5,6} We have identified the subcellular sites of arachidonoyl phospholipids following brief exposure of unstimulated human platelets to [³H]arachidonate. Our findings indicate that

after five minutes of exposure to arachidonate, virtually all of the incorporated [³H]arachidonate is found in the dense tubular system, suggesting an important role for this internal membrane system in arachidonate metabolism in the platelet.

MATERIALS AND METHODS

Isolation of platelets and labeling with [³H]arachidonate. Platelets were isolated from human blood according to the method of Baenziger et al⁷ and resuspended at a concentration of 5×10^8 /mL in platelet wash buffer (26.2 mmol/L sodium phosphate and 6.8 mmol/L potassium phosphate, pH 6.5, 118 mmol/L sodium chloride, 5.6 mmol/L glucose). Four aliquots (0.2 mL) were incubated with 750 nmol/L [5,6,8,9,11,12,14,15-³H]arachidonate (135 Ci/mmol, Amersham, Arlington Hts, IL) to label the platelets to a high specific radioactivity and allow development of an adequate number of grains for quantitative autoradiography; four aliquots were incubated with 3.7 nmol/L [³H]arachidonate and used in assays to determine the amount of [³H]arachidonate removed from platelets during processing of the samples for electron microscopy; four aliquots were incubated in the absence of radiolabeled arachidonate and used to determine background grain density. Following incubation for 5 minutes at 37°C to allow incorporation of exogenous [³H]arachidonate, 0.8 mL of platelet wash buffer was added to each tube and the cells centrifuged at $12,000 \times g$ for ten seconds. The platelets were resuspended in 1 mL of platelet wash buffer containing 1 mg/mL fatty acid free bovine serum albumin (Sigma, St Louis), centrifuged again at $12,000 \times g$ for ten seconds, and resuspended in 0.2 mL of platelet wash buffer without bovine serum albumin. The platelets were incubated for an additional 5 minutes at 37°C and then fixed by the addition of 1 mL of tannic acid-glutaraldehyde (3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, containing 1.5% tannic acid and 2 mmol/L CaCl₂). The fixed platelets were pelleted at $12,000 \times g$ for one minute and resuspended in tannic acid-glutaraldehyde for additional fixation for two hours at room temperature.

Preparation and analysis of autoradiographs. Platelets were processed for electron microscopic autoradiography according to methods developed in our laboratory.⁸ Briefly, the fixed platelets were rinsed with 0.1 mol/L sodium cacodylate buffer, pH 7.4, containing 2 mmol/L CaCl₂, post-fixed with 2% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for one hour at 4°C, rinsed in 0.8% sodium acetate buffer, pH 7.2, and stained en bloc with 0.5% uranyl magnesium acetate in 0.8% sodium acetate buffer overnight at 4°C. The stained platelets were rinsed with acetate buffer, dehydrated rapidly at 4°C with increasing concentrations of acetone, and infiltrated with Spurr's low viscosity epoxy resin.

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Platelets incubated with 750 nmol/L [³H]arachidonate were embedded in Spurr's resin by pelleting the cells in polypropylene capsules containing resin. These platelets were sectioned for analysis with electron microscopic autoradiography. Platelets incubated with 3.7 nmol/L [³H]arachidonate were used to assess preservation of radioactivity during tissue processing. Following each step, platelets were pelleted, and the processing reagent was transferred to a vial. Radioactivity removed from the platelets during each step was quantified with liquid scintillation spectrometry. At the completion of processing, the platelets were dissolved in NCS (Amersham), and radioactivity retained in the cells was determined with liquid scintillation spectrometry.

The chemical composition of radiolabeled material removed during acetone dehydration was determined by thin layer chromatography.⁹ The combined acetone washes of platelets incubated with 750 nmol/L [³H]arachidonate were concentrated into a small volume by evaporation under nitrogen and analyzed by thin layer chromatography in a phospholipid separation system containing CHCl₃:CH₃OH:glacial acetic acid:H₂O (60:30:16:3.2).

Autoradiographs were prepared according to a modification of the flat substrate method of Salpeter and Bachmann,¹⁰ as previously described.^{11,12} Pale gold sections, approximately 100 nm thick, were mounted on collodion-coated slides, shadowed with a thin (5 to 6 nm) layer of carbon, and coated with monolayers of Ilford L-4 nuclear tract emulsion. The slide-mounted sections were stored in light-tight boxes at 4°C for 96 hours. The emulsion was developed in 1.1% p-phenylenediamine and 12.6% sodium sulfite, and fixed in 30% sodium thiosulfate, as previously described.³ Sections were collected on 200-mesh copper grids and examined in a Phillips 200 EM electron microscope at 60 KeV. Sections prepared from unlabeled platelets for assessment of background grain density were placed on the same collodion-coated slides as the radiolabeled tissue sections. Previous studies from our laboratory have demonstrated no significant artifacts due to positive or negative chemography or latent image fading in the autoradiography procedures used in these studies.¹¹

Autoradiographs were analyzed according to the mask overlay method of Salpeter et al.¹³ using the computer program of Land and Salpeter.¹⁴ This method accounts for the problem of image spread in which a grain is formed overlying one compartment when the radiolabeled probe is located in a different compartment. This method uses a mathematical model describing the spread of grains around point sources of radiation and estimates of the probabilities that a point source of radioactivity located in one structure will give rise to grains overlying any other structure. The half-distance (radius of a circle around a point source in which 50% of grains will be contained) for the autoradiographic system used is approximately 150 nm measured in a system with tritium, monolayers of Ilford L-4 emulsion, 100 nm thick sections, and p-phenylenediamine developer.¹³ Although p-phenylenediamine is an inefficient developer (approximately 1 grain per 15 disintegrations),¹⁵ it produces small compact grains that minimize obstruction of underlying fine structure.¹⁶

A total of 150 randomly selected photographs containing 410 platelets and 741 grains was analyzed at a final print magnification of $\times 48,000$. All structures appearing in the electron micrographs were assigned to individual source compartments defined as potentially labeled structures. Source compartments, encompassing the entire photographic area, included specific membranous organelles of platelets, all remaining platelet cytoplasm, and extracellular space between the platelets. The dense tubular system and the open canalicular system of membranes could be readily distinguished by their ultrastructure, but because of their small size and intimate anatomic proximity, the spread of grains resulting from radiolabeled sources in either of the two compartments overlapped to such an

extent that they could not be resolved autoradiographically. Accordingly, these two internal membrane compartments were combined to form a single source compartment referred to as DTS/OCS. Additional source compartments analyzed were the plasma membrane, mitochondria, all platelet granules including dense bodies, all remaining nonmembranous platelet structures, and the extracellular space between platelets.

RESULTS

[³H]arachidonate was taken up rapidly by platelets and incorporated into phospholipids. In separate experiments, we determined that over 90% of esterified radiolabeled arachidonate was present in phospholipids. Platelets incubated with 3.7 nmol/L [³H]arachidonate for five minutes at 37°C incorporated approximately 37% of the exogenous labeled arachidonate. Loss of cell radioactivity during tissue processing for electron microscopy was minimal. As shown in Table 1, 86% of incorporated radioactivity was retained by platelets following tissue processing. Approximately one third of the radioactivity removed during processing was recovered in the initial aqueous fixation steps and presumably reflects removal of radioactivity loosely associated with the platelets rather than extraction of incorporated cellular lipids. Most of the remaining radioactivity removed during processing was recovered during the dehydration and resin infiltration steps involving organic solvents. Thin layer chromatographic analysis of material extracted during dehydration in acetone showed that virtually all radioactivity was present in neutral lipid (data not shown). Thus, the modest amount of radioactivity removed during tissue processing for electron microscopy was predominantly unincorporated radioactivity or radioactivity in neutral lipid. Labeled phospholipids, nearly quantitatively preserved, were spatially fixed during tissue processing. Thin layer chromatographic analysis of the small amount of labeled phospholipid removed during dehydration with acetone revealed that no single phospholipid species was extracted selectively. Because there is little conversion of arachidonate to other fatty acids and the bulk of oxygenated arachidonate metabolites exit the cell, radioactivity retained in platelets was assumed to be [³H]arachidonate. In this regard, Banerjee and Rosenthal showed in human skin fibroblasts incubated 5 minutes with submicromolar concentrations of arachidonate that a maximum of 10% of incorpo-

Table 1. Loss of Radioactivity During Tissue Processing

Step	Percent of Total Radioactivity
Tannic acid—glutaraldehyde + rinse	4.41
Osmium tetroxide + rinse	0.91
Uranyl magnesium acetate + rinse	1.91
Dehydration	
70% acetone	0.47
95% acetone	0.26
100% acetone	3.35
Resin infiltration	
Resin:acetone, 1:1	1.32
100% resin	0.75

Total percent removed, 13.40; Percent retained in tissue, 86.60.

Total dpm retained in tissue, 7.41×10^5 dpm.

Total platelet number in sample, 1×10^8 .

rated arachidonate is elongated to 22:4n-6.¹⁷ Furthermore, at the concentration of exogenous arachidonate we used, arachidonate is rapidly esterified into phospholipids with minimal conversion to icosanoids.⁵

Fig 1 shows the results of analysis of electron microscopic autoradiographs. It shows the size of each source compartment expressed as percent of total photographic area (Fig 1A), the specific activity of each source compartment expressed as grains per square micrometer of structure area

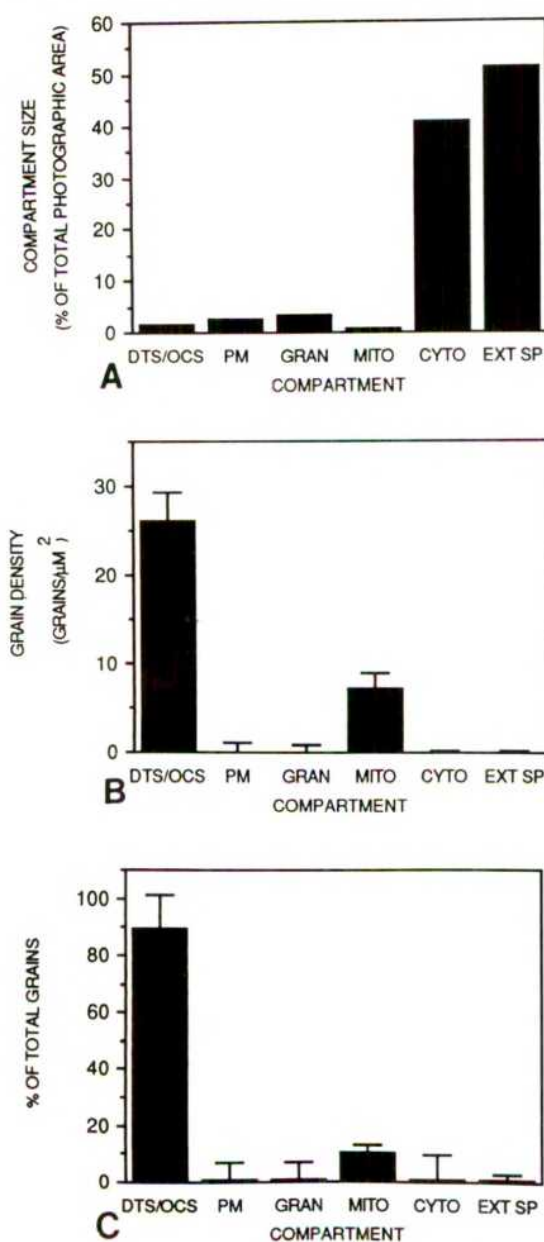


Fig 1. Subcellular distribution of [³H]arachidonate in platelets. DTS/OCS: dense tubular system open canalicular system. PM: plasma membrane. GRAN: granules including granule membranes and dense bodies. MITO: mitochondria. CYTO: all remaining cytoplasmic structures. EXT SP: extracellular space. Background grain density was approximately 0.08 grains/100 μm². Error values shown represent the "standard error" of the Chi-square minimization routine of Land and Salpeter.¹⁴

(Fig 1B), and the percent of total radioactivity contained in each compartment (Fig 1C). Only two structures were labeled. The combined internal membrane compartment composed of the dense tubular system and open canalicular system had a grain density of approximately 26 grains per square micrometer and contained approximately 90% of total platelet radioactivity. Remaining radioactivity was associated with the mitochondrial compartment. Background grain density, determined from sections of unlabeled platelets, was negligible (approximately 0.1 grain/100 μm²).

Figs 2 and 3 show representative low and high magnification electron microscopic autoradiographs of human platelets labeled with [³H]arachidonate. Platelets exhibited typical discoid morphology and contained numerous granules, indicating they were in the unstimulated condition.

DISCUSSION

Quantitative electron microscopic autoradiography is particularly well suited for subcellular localization of arachidonoyl phospholipids. Radiolabeled arachidonate is available with high specific activity, thus minimizing emulsion exposure intervals, and the polyunsaturated acyl moieties are extensively cross-linked by osmium tetroxide, resulting in spatial fixation of arachidonoyl phospholipids, precluding their extraction or intracellular translocation during tissue processing.⁸ This provides a significant advantage over cellular fractionation methods in which hours may elapse between the end of an interval of arachidonate incorporation and the collection of fractions for analysis of arachidonate content. With electron microscopic autoradiography, there is no such period for redistribution of arachidonate among subcellular membranes, because fixation preserves the distribution of

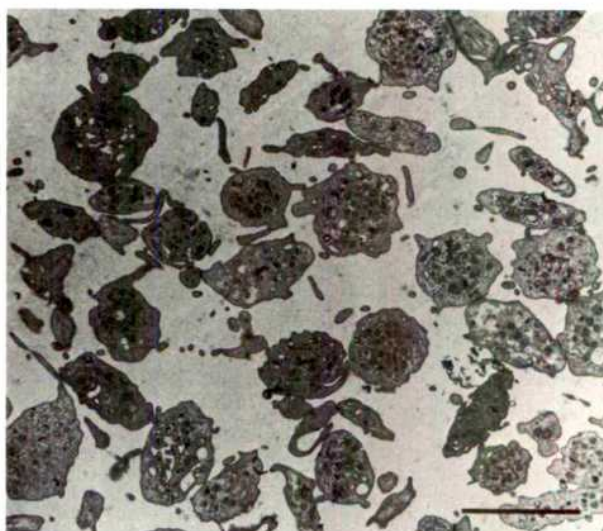


Fig 2. A low magnification electron microscopic autoradiograph showing the distribution of grains (dense black structures) in platelets incubated with 750 nmol/L [³H]arachidonate for 5 minutes. Platelets exhibit normal discoid morphology and contain numerous granules indicating they have not become activated. Original magnification × 6300; current magnification × 4,284. Bar = 3 μm.

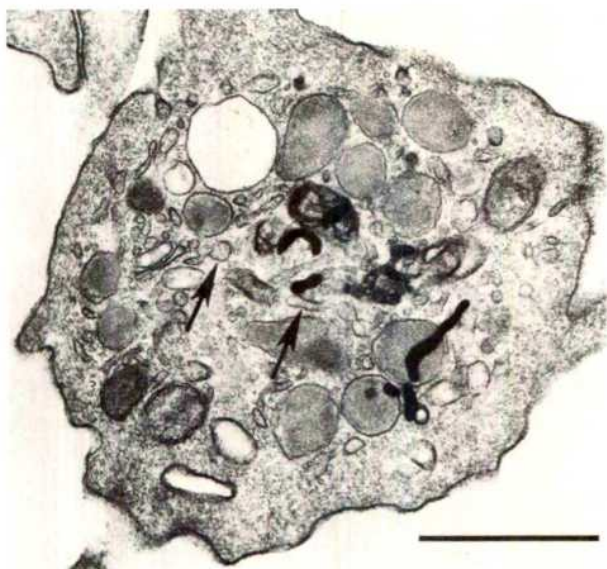


Fig 3. A high magnification autoradiograph showing the relative size of grains (dense black structures) and the membranous components of the dense tubular system and open canalicular system (arrows). Original magnification $\times 35,000$; current magnification $\times 23,800$. Bar = $1\ \mu\text{m}$.

arachidonate immediately following the interval of incorporation.

The results of the present study demonstrate that brief (five minutes) exposure of human platelets to physiologic concentrations of arachidonate *in vitro* results in rapid and selective labeling of an intracellular membrane compartment that includes the dense tubular system and open canalicular system. This combined membrane compartment comprised only 1.5% of total photographic area but contained approximately 90% of labeled arachidonate. Remaining arachidonate radioactivity was localized to mitochondria. The significance of the low level of incorporation of [^3H]arachidonate in mitochondria in our experiments is unknown. The plasma membrane was not labeled. Because arachidonate has been shown to rapidly translocate between subcellular membranes,³ the subcellular distribution of arachidonate is likely to be different following longer incubations of platelets with arachidonate. For example, in a study involving a one-hour exposure of human platelets to [^{14}C]arachidonate (0.4 nmol/L), Lagarde et al separated surface and intracellular membranes by high voltage free flow electrophoresis and found significant amounts of [^{14}C]arachidonate in both surface and intracellular membranes.¹⁸ Also, both Lagarde et al¹⁸ and Perret et al¹⁹ found endogenous arachidonate in isolated plasma membranes from human platelets. In light of our findings, it appears that arachidonate is initially incorporated in the dense tubular system with subsequent translocation of an arachidonate pool to the plasma membrane. In an electron microscope autoradiography study of mouse fibrosarcoma cells labeled with [^3H]arachidonate, it was shown that the amount of arachidonate in the plasma membrane increased four- to eightfold as the incubation time with [^3H]arachidonate was extended from fifteen minutes to two hours, indicating arachidonate movement into the plasma

membrane.³ Thus, the difference in incubation time with radiolabeled arachidonate may explain the presence of arachidonate in the plasma membrane in Lagarde's studies with a one-hour arachidonate exposure and the absence in our studies (five-minute incubation). This would also explain the presence of endogenous arachidonate in isolated plasma membranes. With regard to arachidonate compartmentation within phospholipids following brief exposure to arachidonate, it has been shown that after a fifteen-minute incubation, greater than 60% of incorporated arachidonate is esterified into phosphatidylcholine.²⁰

Although the tubulovesicular membranes of the dense tubular system and open canalicular system could be distinguished by their different ultrastructures, their intimate anatomic proximity and the spread of grains precluded autoradiographic resolution of the two compartments. However, the open canalicular system is continuous with the plasma membrane,^{21,22} which we have shown was not labeled. Furthermore, studies by Pagano et al²³⁻²⁵ using fluorescent phospholipids demonstrate that phospholipids within continuous membrane systems are freely diffusible. Based on their data on the rate of phospholipid diffusion within membranes, grains associated with the plasma membrane would be present if the open canalicular system were highly labeled.

Our data indicate that arachidonate was incorporated into phospholipids in the dense tubular system rather than in the open canalicular system. The dense tubular system is considered to be the endoplasmic reticulum of mature platelets. This internal membrane system contains peroxidase and glucose-6-phosphatase, enzymes present in the rough endoplasmic reticulum of megakaryocytes.²⁶⁻²⁸ We have recently shown that the endoplasmic reticulum is a major storage site for arachidonoyl phospholipids in a mouse fibrosarcoma line.³ Moreover, analysis of major membrane pools of rat liver has shown that the endoplasmic reticulum is enriched in arachidonate relative to the plasma membrane.²⁹ These findings are compatible with our results, which suggest that arachidonate is rapidly and selectively incorporated into phospholipid in the dense tubular system of the platelet. In nucleated cells, the nuclear membrane appears to be a kinetically preferred site for the incorporation of arachidonate into phospholipids and may also serve as a reservoir for arachidonate released for eicosanoid production.³ Using a mutant cell line devoid of arachidonoyl-CoA synthetase activity, we have demonstrated that incorporation of arachidonate into the nuclear membrane is impaired.³ Despite normal cyclooxygenase and phospholipase activities and abundant intracellular arachidonate, mutant cells synthesize significantly smaller amounts of eicosanoids than control cells.¹⁻³ Thus, agonist-mediated eicosanoid production may depend on appropriate subcellular compartmentalization of arachidonoyl phospholipids, presumably controlled by the high affinity arachidonoyl-CoA synthetase. This compartmentation concept is supported by observations that enzymes of eicosanoid synthesis such as cyclooxygenase and prostaglandin I_2 synthetase reside in the nuclear membrane and endoplasmic reticulum.³⁰⁻³² Our findings that the dense tubular system becomes rapidly and selectively labeled with newly incorporated arachidonate suggest this membrane

system may function in a manner analogous to the nuclear membrane and endoplasmic reticulum of nucleated cells. In fact, Carey et al³³ have shown, using platelet membrane fractions isolated by high voltage free flow electrophoresis, that platelet cyclooxygenase and thromboxane synthetase activities are found predominantly in an intracellular membrane fraction with little of either activity in surface membrane fractions, granules, or cytosol. The localization of arachidonate metabolizing enzymes in their studies resembles the localization of incorporated arachidonate in our experiments. In an earlier study, Gerrard et al demonstrated that of several subcellular platelet fractions, the one most enriched in elements of the DTS was the most efficient in

converting ¹⁴C-arachidonate to icosanoids.³⁴ Thus, present evidence indicates that in platelets arachidonate is incorporated into phospholipids in the dense tubular system and, when liberated upon platelet activation, the free arachidonate is converted to thromboxane A₂ by enzymes in the same membrane compartment.

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Studies With a Monoclonal Antibody Against Activated Platelets: Evidence That a Secreted 53,000-Molecular Weight Lysosome-like Granule Protein Is Exposed on the Surface of Activated Platelets in the Circulation

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To define the role of activated platelets we have attempted to prepare monoclonal antibodies specific for activated platelets. The IgG2b antibody of one of the clones, designated 2.28, was studied in more detail. Native platelets from normal individuals bound 650 ^{125} I-2.28 molecules/platelet, whereas thrombin-activated platelets bound 12,600 molecules/platelet with high affinity (4.6 nmol/L). Immunoelectrophoretic analysis revealed that 2.28 reacted with a 53,000-mol wt protein. Immunocytochemistry showed that the antigen is located in a special subclass of platelet granules in unstimulated platelets and is exposed on the surface of thrombin-activated platelets. Double-labeling studies with immunogold labels disclosed simultaneous localization of 2.28 binding sites and cathepsin D in the same granules both in megakaryocytes and endothelial

cells, thereby indicating that the antigen may be localized in lysosomes. By using flow cytofluorometry, *in vivo* platelet activation was studied in patients undergoing cardiac surgery with cardiopulmonary bypass. Increased numbers of platelets that expressed the 2.28 antigen on their surface were observed after extracorporeal perfusion. The percentage of 2.28-positive platelets in the circulation was $3.9\% \pm 2.7\%$ (SD) in controls ($n = 20$), $5.5\% \pm 3.0\%$ in patients ($n = 10$) before cardiopulmonary bypass surgery, $24.6\% \pm 13.5\%$ after the bypass, and 8.5% in two patients with acute deep venous thrombosis. These data indicate that 2.28 may serve as a useful probe of *in vitro* and *in vivo* platelet activation.

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ACTIVATION OF HUMAN BLOOD platelets is associated with changes in platelet morphology, biochemistry, and membrane composition. The events result in binding or increased surface expression of several endogenous platelet proteins and plasma proteins. A number of the biologic activities of platelets in hemostasis and thrombosis have been attributed to these changes in membrane composition. Stimulated platelets bind von Willebrand factor,¹⁻³ fibrinogen,^{4,6} fibronectin,⁷ and thrombospondin,^{8,9} interactions involved in platelet adhesion and aggregation. Other changes in the platelet membrane such as the exposure of a procoagulant surface that promotes prothrombin and intrinsic factor X activation^{10,11} and the expression of binding sites for high-molecular weight (mol wt) kininogen,¹² factor XIa,¹³ factor XIIIa,¹⁴ activated protein C,¹⁵ and plasminogen¹⁶ may be important for the role of activated platelets in coagulation and fibrinolysis.

Blood tests reflecting activation of platelets are potentially useful in understanding the physiological role of activated platelets and may be useful in evaluating patients with thrombotic diseases. To develop methods for the detection of activated platelets in the circulation, we used the hybridoma technique¹⁷ to produce murine monoclonal antibodies (MoAbs) that react with activated platelets but do not react with resting platelets. This report describes the characteristics of one of the most potent MoAbs.

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METHODS

Platelet preparation. Freshly fixed platelets were prepared by collecting blood by venipuncture in 3 vol of paraformaldehyde (final concentration, 1% wt/vol) in phosphate-buffered saline (PBS). The fixed platelets were washed twice and resuspended in PBS.

Thrombin-activated platelets were prepared by collecting blood in 0.1 vol 135 mmol/L trisodium citrate. The platelets were washed three times in 5 mmol/L EDTA-PBS, and afterwards thrombin, to a final concentration of 5 U/mL, was added to the platelet suspension and incubated without stirring for two minutes. The activated platelets were washed (for the preparation of MoAbs) or fixed and washed as described earlier (for immunofluorescence microscopy, flow cytofluorometry, and binding studies).

Antibody production. BALB/c mice were immunized subcutaneously on day 1 with 5×10^9 thrombin-activated washed platelets, suspended in 300 μ L Tris-buffered saline and 1 mmol/L EDTA, and mixed with complete Freund's adjuvant and with activated platelets without Freund's adjuvant on days 21 and 28. Mice were boosted with 10×10^9 activated platelets on day 35, three days before removal of the spleens. Spleen cells were fused according to Köhler and Millstein¹⁷ with cells of the mouse myeloma cell line X63.Ag 8.653,¹⁸ a hypoxanthine phosphoribosyl transferase-deficient line that synthesizes no immunoglobulin. Four hybridoma supernatants (2.2, 2.28, 2.45, and 2.47) were identified that bound to thrombin-activated platelets but did not react with resting platelets as assessed by fluorescence microscopy.¹⁹ These hybridomas were subcloned by limiting dilution. Ascitic fluid rich in 2.28 antibody was prepared by intraperitoneal injection of pristane-treated BALB/c mice with the hybridoma cell line 2.28.

Antibody purification. Ascites was applied to a 0.9×12.0 -cm column of protein A-Sepharose CL-4B that had been equilibrated with 0.1 mol/L sodium phosphate buffer, pH 8.0. The column was eluted with the phosphate buffer until the optical density of the eluate returned to base line, after which elution was accomplished with 0.1 mol/L acetic acid and 0.15 mol/L NaCl, pH 2.5. Protein elution was monitored by optical density at 280 nm. Fractions were pooled and dialyzed against PBS, pH 7.3. This purification yielded an antibody preparation that was >95% pure as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antibody iodination. The purified MoAb 2.28 was radiolabeled with ^{125} I by using the chloramine-T method.²⁰ Noncovalently linked ^{125} I was removed by dialysis overnight at 4°C against 0.05 mol/L

Tris-HCl and 0.1 mol/L NaCl, pH 7.0. The specific activity was 530 cpm/ng, and greater than 96% of the radioactivity was precipitated by 10% trichloroacetic acid.

Immunocytochemistry. Immunofluorescence studies were performed on paraformaldehyde-fixed resting platelets, fixed thrombin-activated platelets, and sections of about 200 nm of platelets and endothelial cells as described.²¹

Immunoelectron microscopy was performed as described previously.²²⁻²⁴ Ultrathin cryosections were incubated with antibody 2.28 and labeled with colloidal gold particles conjugated to protein A. After immunolabeling the sections were stained with uranyl acetate and embedded in methylcellulose according to Tokuyasu.²⁵ In some studies we used a rabbit antihuman placental cathepsin D, an antibody that has been characterized before.²⁶⁻²⁸

Platelet aggregation. Platelet aggregation studies were performed at 37°C in a Payton dual-channel aggregation module (Payton Associates Inc, Buffalo) with 5 μ mol/L adenosine diphosphate (ADP) (Dade Diagnostics Inc, Aquada, PR), 5 μ mol/L epinephrine (Dade), 1.5 mmol/L arachidonic acid (Bio-Data Corp, Hatboro, PA), 10 μ mol/L ionophore A23187 (Boehringer GmbH, Mannheim, FRG), and 1 mg/mL ristocetin (H. Lündbeck & Co, Copenhagen). The aggregation studies were performed in platelet-rich plasma (0.5 mL, 250,000 platelets/ μ L) after preincubation with 10 to 40 μ L 2.28 ascites for 15 minutes at 37°C.

Electrophoretic analysis of platelet antigen recognized by 2.28. Human blood platelets isolated from 100 mL blood were washed three times and suspended in 2 mL of 5 mmol/L EDTA-PBS. Secretion of proteins was induced by adding 10 units thrombin (Sigma Chemical Co, St Louis) at 37°C. After two minutes 20 units of hirudin (Sigma) was added. The suspension was then centrifuged, and the supernatant was used for further electrophoretic analysis.

One-dimensional SDS slab gel electrophoresis was performed according to Laemmli²⁹ on a polyacrylamide gradient of 3% to 30% with a 3% stacking gel. Protein blotting onto nitrocellulose paper was performed with the Western blot technique according to Towbin et al³⁰ with minor modifications using nitrocellulose sheets (Schleicher & Schüll, FRG, BA 85, 0.45 μ m) and electrophoresis for 15 hours at 12 V/cm. Immediately after transfer, the nitrocellulose sheets were immersed in 5% bovine serum albumin in Tris-buffered saline (0.15 mol/L NaCl, 10 mmol/L Tris-HCl, pH 7.4) for 16 hours at 4°C to saturate additional protein-binding sites. The sheets were rinsed six times in Tris-buffered saline and 0.1% Tween 20 and incubated for two hours at 20°C with the radiolabeled monoclonal antibody 2.28 (0.5 μ g/mL diluted 1:100 in Tris-buffered saline with 5% bovine serum albumin). The sheets were then washed in Tris-buffered saline and 0.1% Tween 20 (six changes in one hour) and dried between cellophane sheets (Bio-Rad Laboratories, Richmond, CA). Autoradiography of the dried gels was performed at -70°C by using Kodak X-Omat AR5 (Eastman Kodak Co, Rochester, NY) x-ray film and DuPont Cronex Lightening Plus intensifying screens (DuPont Instruments, Wilmington, DE). A mixture of low-mol wt proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) and high-mol wt proteins (Bio-Rad) was analyzed on every gel as mol wt markers. For blocking experiments, the sheets were incubated with ¹²⁵I-2.28 and an excess (200-fold) of nonradiolabeled 2.28 or non-specific mouse ascites from X63.Ag 8.653 myeloma cell lines.

Electrophoretic analysis of the endothelial cell component recognized by 2.28. Human vascular endothelial cells were isolated from umbilical veins and cultured in 75-cm² flasks as described by Jaffe et al³¹ with some minor modifications.³² The cells were identified by their typical characteristics.³¹ Cells from the second passage were cultured, and after the cells had reached confluency, they were washed twice with PBS and incubated for 24 hours with leucine-depleted Ham's F-10 medium (Flow Laboratories, Irvine, UK)

supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), amphotericin B (5 μ g/mL), ultraser (2% vol/vol final concentration), and 250 μ Ci ³H-leucine (Amersham Corp, Buckinghamshire, England). The cells were washed twice with PBS and solubilized for one hour at 4°C with 10 mmol/L triethanolamine and 150 mmol/L NaCl buffer, pH 7.8, containing 1% (vol/vol), nonidet NP-40 (Sigma), and the following protease inhibitors: 4 mmol/L phenylmethylsulfonylfluoride, 20 mmol/L D-aminobenzamide, 0.2 mg/mL soybean trypsin inhibitor, 2 mmol/L iodoacetamide, 2 mmol/L ϵ -aminocaproic acid, and 5 mmol/L Na₄ EDTA. The lysate was centrifuged for five minutes (10,000 g) at room temperature to remove undissolved material.

The cell lysate (1 mL from approximately 10⁶ cells) was passed over a CNBr-Sepharose 4B (Pharmacia) column (0.6 $\times$ 3 cm) to which 2.1 mg of normal goat IgG had been covalently coupled according to the manufacturer's instructions. Protein A-Sepharose CL4B (Pharmacia) was rehydrated in 0.1 mol/L phosphate buffer, pH 8.0, supplemented with 0.05% (vol/vol) Tween 20, 0.02% (wt/vol) Na₃N₃, and 0.1% (wt/vol) bovine serum albumin. After the Sepharose had been washed twice with the buffer, approximately 3 mg of protein A-Sepharose was incubated overnight at 4°C with either 50 μ L normal mouse serum or 50 μ L of 2.28 ascites fluid. The Sepharose beads were incubated for three hours at room temperature with the flow-through fraction of the cell lysate. The beads were washed eight times by centrifugation with 10 mmol/L triethanolamine and 500 mmol/L KCl buffer, pH 7.8, containing 1% (vol/vol) NP40 and then twice with PBS. The bound protein was extracted from the Sepharose beads by boiling for five minutes in the presence of 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.025% bromophenol blue, and 5% 2-mercaptoethanol in 0.125 mol/L Tris-HCl, pH 6.8. The extract was analyzed by SDS-PAGE in slab gels (3% to 30%). After electrophoresis gels were treated with EN³HANCE (New England Nuclear, Boston) according to the manufacturer's instructions and dried, and subsequently an autoradiogram was made.

Binding of ¹²⁵I-antibody to platelets. Binding of ¹²⁵I-2.28 to paraformaldehyde-fixed unstimulated and thrombin-stimulated platelets was studied by adding varying concentrations of ¹²⁵I-2.28 to the washed platelet suspensions in such a way that the final platelet

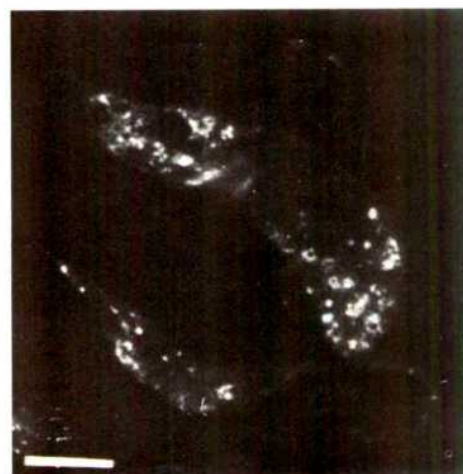


Fig 1. Immunofluorescent staining for 2.28 binding sites in 200-nm cryosections of endothelial cells. The labeling reveals a punctate, intracellular pattern, which indicates localization in granules. (Original magnification $\times$ 1,500; current magnification $\times$ 1,125; bar, 10 μ m.)

concentration was 10^8 platelets/mL and the final volume 120 μ L. After an incubation for 120 minutes at 20°C, a 100- μ L aliquot was layered on top of 50 μ L phthalate and centrifuged at 12,000 g for two minutes. After removing the supernatant, centrifuge tube tips were cut off and the bound radioactivity determined in a gamma counter. Nonspecific binding was determined by adding a 200-fold excess of unlabeled antibody to the incubation mixtures. This value was subtracted from the bound counts per minute to yield total specific bound counts per minute. The binding assays were performed in duplicate. The binding affinity of antibody 2.28 and the total number of antibody molecules bound per platelets were calculated by using the HYPER computer program as described by Cleland.³³

Flow cytometry. Measurements were carried out in a cytofluorograph 50-H (Ortho Instruments, Westwood, MA) using laser light of 488 nm at an intensity of 250 mW. Fluorescence and scatter signals were standardized daily by using fluorescent monodisperse carboxymethylated microspheres (diameter, 2.04 μ m $\pm$ 0.02 SD; Polysciences, Inc, Warrington, PA). Size was measured by

using the blue forward scatter, and gating was set such that 90% of the particles in a sample were accepted for fluorescence analysis. Freshly paraformaldehyde fixed unstimulated platelets and fixed thrombin-activated platelets (4×10^7 platelets in 200 μ L EDTA-PBS) were incubated with 2 μ g purified 2.28 (in 200 μ L EDTA-PBS) for two hours at 20°C. After incubation the platelets were washed three times and incubated with 50 μ L of diluted (1:50) fluorescein isothiocyanate (FITC)-labeled goat antimouse (GAM) IgG (Central Laboratory of Blood transfusion, Amsterdam) for 30 minutes at 20°C. The platelets were then washed twice with PBS and resuspended in 1 mL PBS. A total of 10^5 platelets of each sample was analyzed at a rate of 2,000 platelets per second. A control specimen containing the FITC-coupled antibody with a purified aspecific MoAb and a control specimen containing 2.28 without the FITC-coupled antibody served as negative controls. Flow cytometry results were compared with visual fluorescence microscopy and found to be concordant. Twenty healthy controls and 12 patients were studied.

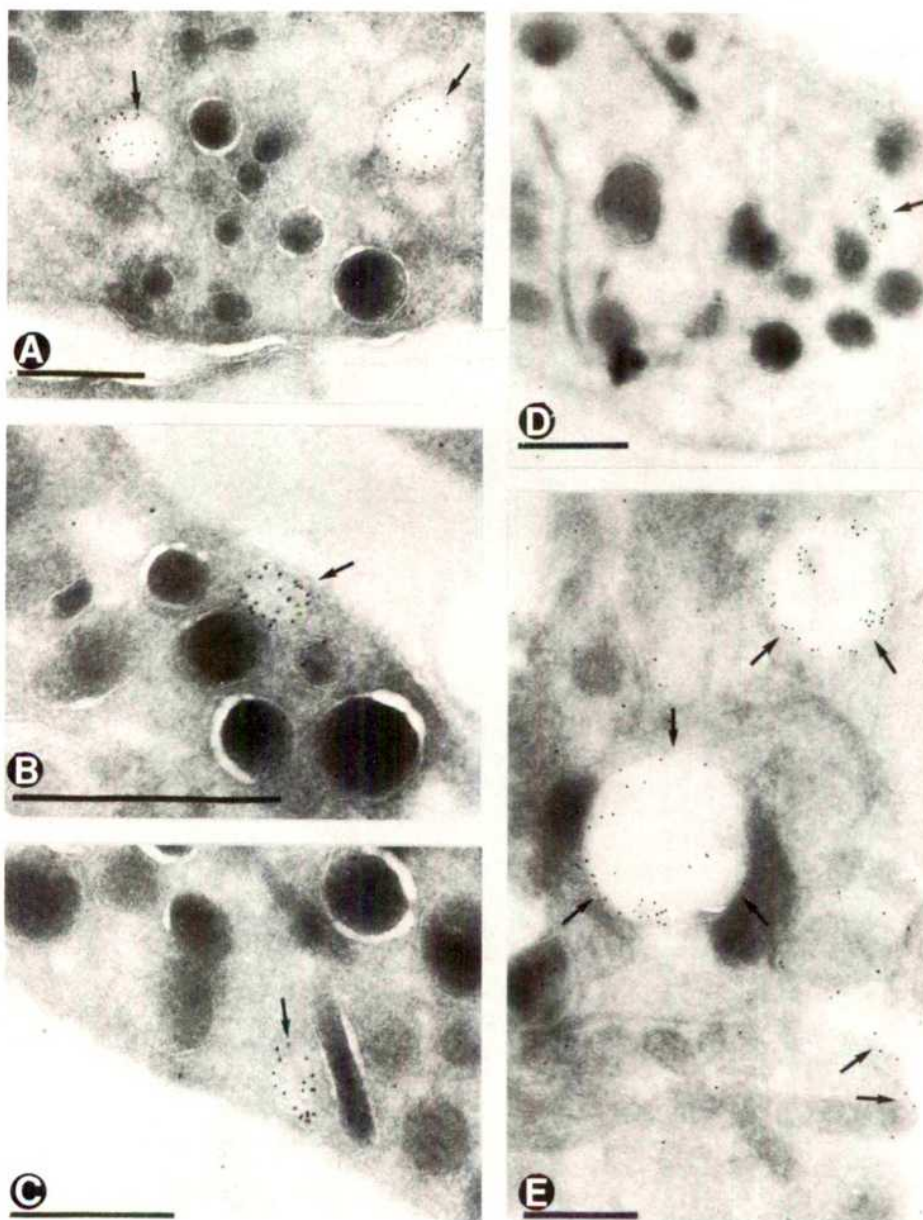


Fig 2. Localization of 2.28 binding sites by electron microscopic immunocytochemistry with colloidal gold particles in unstimulated (A to D) and thrombin-stimulated (E) human blood platelets. (A to D) Label is present in granules with moderate to low electron density but not in the cytoplasmic matrix, mitochondria, or on the cell membrane. (E) In stimulated platelets gold particles are present in the surface-connected canalicular system. A few particles can be seen on the platelet plasma membrane at the right bottom. Colloidal gold particles, 8 nm. (Original magnifications: A $\times 44,000$, B $\times 95,000$, C $\times 61,500$; D $\times 40,000$; E $\times 41,000$; current magnifications: A $\times 33,000$, B $\times 71,250$, C $\times 46,125$, D $\times 30,000$, E $\times 30,750$; bar, 0.5 μ m.)

Other methods. Measurement of β -thromboglobulin was performed by using a commercially available radioimmunoassay (Radiochemical Centre, Amersham, UK).

Patient studies. Ten patients were studied who underwent surgery for coronary bypass grafting ($n = 8$) or mitral valve replacement ($n = 2$). The mean age of the patients was 64 years. Surgery was carried out under moderate hypothermic conditions (29° to 27°C). A Bently Bos bubble oxygenator was used, and the mean duration of extracorporeal circulation was 92 minutes with a range of 64 to 148 minutes. Heparin, 300 U/kg, was administered initially, and 100 U/kg was given each hour of extracorporeal perfusion. Protamine sulfate was administered at termination of the bypass in a ratio of 1 mg/100 U of heparin given. A blood sample was drawn via a catheter immediately before perfusion and 20 minutes after cessation of bypass.

Two patients were studied with acute deep venous thrombosis. The diagnosis was made by impedance plethysmography. Blood samples were drawn at presentation by venipuncture.

Twenty normal individuals (aged 22 to 48) volunteered as controls. None of the controls had used any medication for at least 14 days before the investigation.

RESULTS

Using immunofluorescence as a screening test, we identified four MoAbs that bound to thrombin-activated platelets but did not react with resting platelets. The antibodies, designated 2.2, 2.28, 2.45, and 2.47, were shown to be IgG1, IgG2b, IgG1, and IgM respectively by Ouchterlony immunodiffusion analysis. Ascites fluid obtained from mice injected with cells obtained from cell line 2.28 was used for purification of 2.28 by protein A-Sepharose affinity chromatography. Purified 2.28 did not agglutinate platelets, nor did it inhibit ADP-, epinephrine-, thrombin-, collagen-, ristocetin-, or ionophore A23187-induced platelet aggregation.

Immunocytochemical localization. Immunofluorescence studies on cryosections of washed platelets (data not shown) and endothelial cells (Fig 1) revealed a dotlike labeling pattern for 2.28, with no fluorescence of the cell membrane, thereby suggesting that the antigen was localized in granules. To visualize the 2.28 antigen, we used protein A-colloidal gold for immunoelectron microscopy. These studies confirmed that the label was localized in specific platelet granules (Fig 2A to 2D) in unstimulated platelets. The intragranular distribution of the label was diffuse. The label was found in granules with moderate to low electron density but not in the cytoplasmic matrix, mitochondria, or on the platelet membrane. α -Granules were negative. In platelets stimulated with thrombin before fixation, the label was found in the surface-connected canalicular system and on the platelet membrane (Fig 2E). The label was also present in specific granules of endothelial cells. The distribution of the label suggested that the antigen was localized in lysosomes. To prove this localization we investigated simultaneously with a double-labeling technique the distribution of the 2.28 antigen and the lysosomal enzyme cathepsin D. Figure 3 shows that both labels were present in the same granules both in megakaryocytes and in endothelial cells.

Identification of the antigen recognized by 2.28. The supernatant of thrombin-activated platelets was subjected to electrophoresis in a polyacrylamide gel after solubilization in SDS, subsequently blotted on nitrocellulose, and incubated with radiolabeled 2.28. Autoradiography (Fig 4) revealed that ^{125}I -2.28 bound to a protein with an apparent mol wt of 53,000. A minor radiolabeled protein with a mol wt of 40,000 was also present. It is unknown thus far whether the minor band represents a cleavage product induced by thrombin activation of the platelet or another form of the protein.

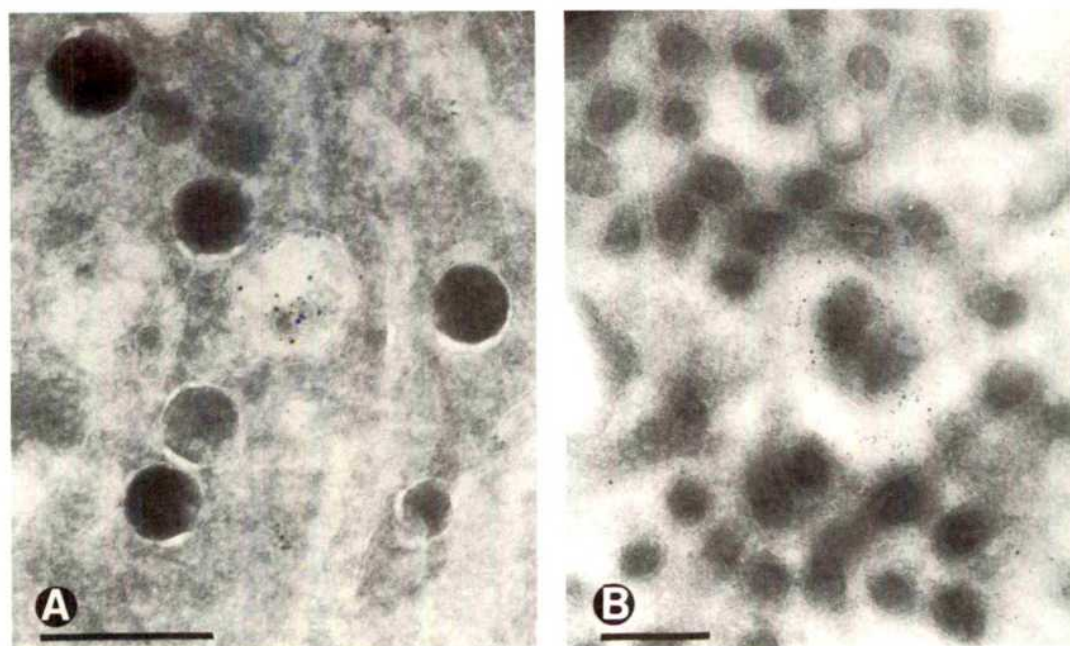


Fig 3. Simultaneous electron microscopic immunocytochemical localization of 2.28 binding sites (6-nm gold particles) and cathepsin D (10-nm gold particles) in the same granule in (A) megakaryocytes and (B) endothelial cells. (Original magnifications: A $\times 53,000$, B $\times 31,000$; current magnifications: A $\times 45,050$, B $\times 26,350$; bar, $0.5 \mu\text{m}$.)

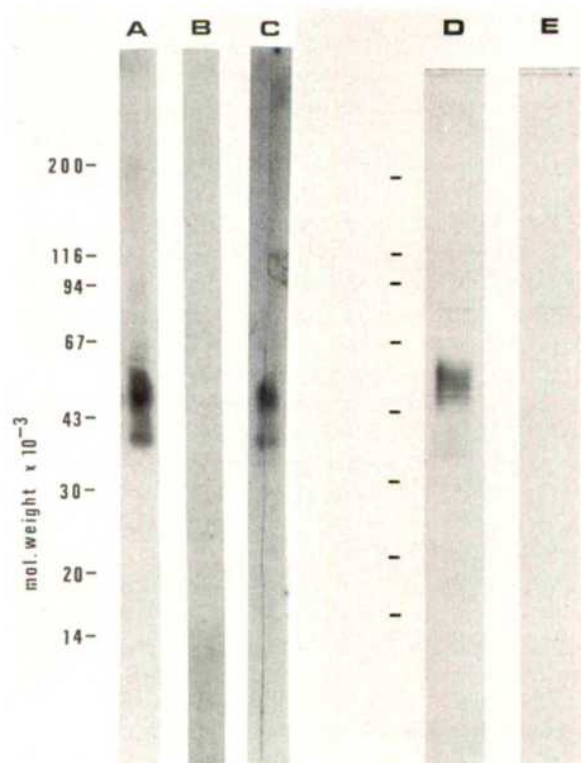


Fig 4. Analysis of platelet and endothelial proteins that bind to 2.28. (Lane A) Autoradiogram of proteins secreted by thrombin-stimulated platelets, subsequently electrophoresed in 3% to 30% polyacrylamide gel, blotted, and incubated with ^{125}I -2.28. Lane A shows a major band of 53,000 and a minor band of 40,000. Lane B and C show blocking experiments. The binding of ^{125}I -2.28 could be blocked by an excess (200-fold) of nonradiolabeled 2.28 (lane B), and nonspecific mouse ascites had no effect (lane C). Lane D shows the ^3H -leucine-labeled extract of endothelial cells immunoprecipitated by 2.28, and lane E shows the control using a nonspecific mouse IgG monoclonal antibody.

These data were obtained by electrophoresis under nonreducing conditions. When the experiments were performed under reducing conditions, ^{125}I -2.28 did not bind, presumably because the 2.28-binding site was disrupted by β -mercaptoethanol.

We also used another approach to identify the component recognized by 2.28. Cultures of human endothelial cells were labeled with ^3H -leucine, and the endothelial proteins were solubilized and immunoprecipitated. The immunoprecipitate was solubilized in SDS, electrophoresed in a polyacrylamide gel under reducing conditions, and visualized by fluorography. Figures 4D and E show that 2.28 recognizes a protein with a mol wt of 53,000 that is synthesized by human endothelial cells. Immunoprecipitation studies with the medium of endothelial cells revealed that the antigen was not excreted in the medium.

Binding of ^{125}I -2.28 to platelets. Binding studies indicated that there was a minimal specific binding of ^{125}I -2.28 to fixed unstimulated platelets. ^{125}I -2.28 bound to fixed thrombin-activated platelets, and time course studies (data not shown) indicated that equilibrium was established after two hours' incubation. Binding was saturable, and analysis (Fig

5) of the binding data indicated that ^{125}I -2.28 bound with high affinity (4.6 nmol/L). Fixed unstimulated platelets bound 650 ^{125}I -2.28 molecules per platelet, and fixed thrombin-activated platelets bound 12,600 ^{125}I -2.28 molecules per platelet.

Analysis of activated platelets by flow cytometry. We studied the number of circulating 2.28-positive platelets by flow cytometry. Figure 6 shows representative fluorescence profiles and cumulative distribution curves of unstimulated and thrombin-activated platelets labeled with 2.28-FITC-GAM.

The percentage of 2.28-positive cells was calculated from cumulative distribution curves. Each sample was analyzed in three ways, which resulted in three cumulative curves: (a) a curve (+2.28, +FITC) showing the fluorescence of cells labeled with 2.28 and FITC-GAM, (b) a curve (-2.28, +FITC) showing the fluorescence of cells obtained by addition of a nonspecific MoAb and FITC-GAM, and (c) a curve showing the fluorescence obtained by the addition of 2.28 alone. Both latter curves represent basic fluorescences, and since these curves were similar in our studies, it was not necessary to correct the data for the aspecific fluorescence caused by FITC-GAM alone. The percentage of 2.28-positive cells in a sample was calculated as the distance (percentage) between the point on the second curve (-2.28, +FITC) at the 99th percentile and the point on the first curve (+2.28, +FITC) in the same channel (Fig 6D).

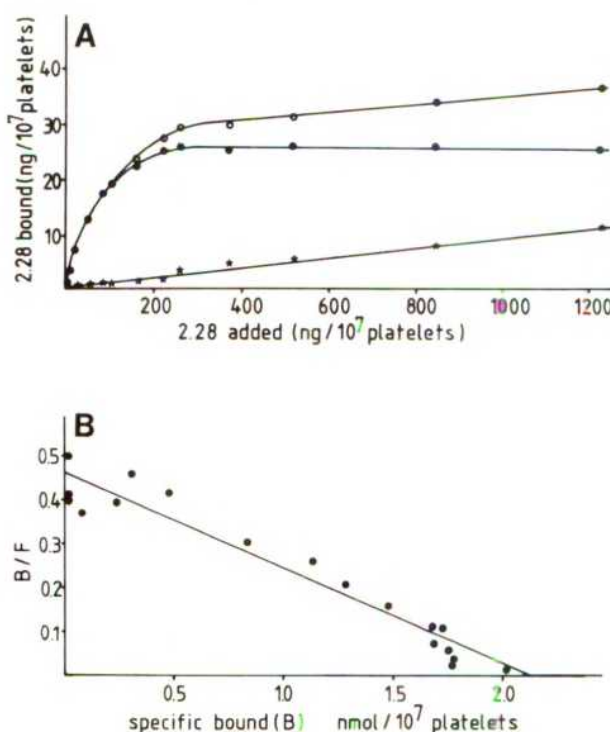


Fig 5. Binding of ^{125}I -2.28 to thrombin-stimulated platelets. ^{125}I -2.28 was added in increasing concentrations to platelets and incubated for two hours at 22°C . Specific binding ($\bullet$) was calculated as the difference between total ($\circ$) and nonspecific binding (Δ). A Scatchard plot of the specific binding is shown in B.

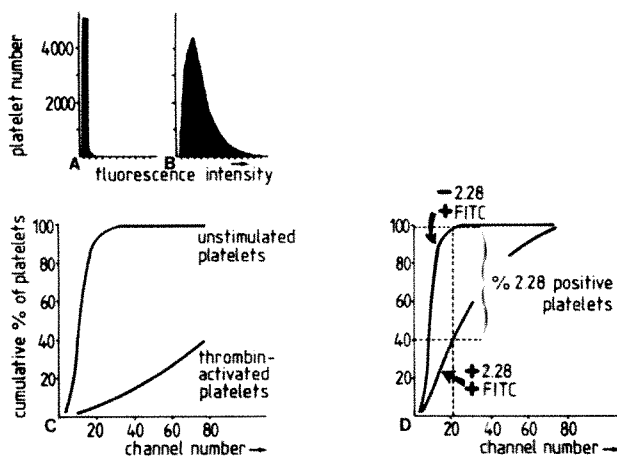


Fig 6. Flowcytometric analysis of 2.28 binding to unstimulated platelets (A) and thrombin-activated platelets (B). Each histogram shows the fluorescence of 100,000 platelets. A cumulative histogram (C) from the fluorescence profiles shown in A and B was obtained by adding the number of platelets in each channel. In a cumulative distribution curve, each point represents the percentage of platelets with fluorescence less than or equal to the channel number. (D) An example of the calculation of the number of 2.28-positive platelets as described in Results.

The percentage of 2.28-positive platelets in the circulation was $3.9\% \pm 2.7\%$ (SD) in healthy controls ($n = 20$). In vitro stimulation with 5 units thrombin resulted in $76.1\% \pm 6.4\%$ (SD) 2.28-positive platelets ($n = 7$). Patients undergoing cardiopulmonary bypass surgery ($n = 10$) had $5.5\% \pm 3.0\%$ (SD) 2.28-positive platelets immediately before extracorporeal perfusion and $24.6\% \pm 13.5\%$ (SD) after the bypass. There was a correlation of .82 with the plasma levels of β -thromboglobulin that increased from 78.9 ± 32.7 (SD) ng/mL before to 645.9 ± 318.0 (SD) ng/mL after extracorporeal perfusion. No correlation was found between the number of 2.28-positive cells and the time of bypass. Two patients with acute deep venous thrombosis had increased levels of 2.28-positive platelets of 7% and 10% respectively.

DISCUSSION

In the present study we characterized a murine MoAb, designated 2.28, that binds to a protein expressed on the surface of human blood platelets after activation. The IgG2b antibody identifies a protein with a mol wt of 53,000 that is localized in lysosomelike granules in unstimulated platelets. After platelets have been stimulated, the protein is secreted via the surface-connected canalicular system and is exposed on the platelet membrane. The protein recognized by 2.28 is also present in granules of endothelial cells, and by using immunoprecipitation studies of endothelial cell cultures we could demonstrate that the protein is synthesized by human endothelial cells. Double-labeling experiments showed that the protein, recognized by 2.28, and cathepsin D were localized in the same granules, thereby indicating that the protein is localized in lysosomes. The identity and function of the reactive antigen is unknown thus far. Its mol wt does not agree with the characteristics of the few proteinases of platelet lysosomes described previously in the literature. A

mol wt of 33,000 has been reported for platelet cathepsin D, 60,000 for cathepsin E, 40,000 for trypsin, 32,000 for a chymotrypsinlike enzyme, and 26,000 for platelet elastase.^{34,35} The function in hemostasis of this protein could not be clarified by using the antibody 2.28. The antibody did not affect platelet aggregation induced by several stimuli.

The binding of radiolabeled 2.28 to thrombin-activated platelets was saturable. Fixed resting platelets bound ~650 molecules of 2.28 per platelet, and fixed thrombin-activated platelets bound ~12,600 molecules per platelet. The low level of binding in native platelets may be due to a low number of binding sites for 2.28 on the total population of native platelets or may be due to heterogeneity of the platelet population, that is, a high number of binding sites on a small number of platelets. To study these two hypotheses we used flow cytofluorometry, a technique that enables measurement of the expression of binding sites for an antibody in each cell separately. The results of the immunofluorescence analysis indicated that immediately fixed native platelets were heterogeneous, a small subpopulation of platelets (4%) expressed the antigen on the surface, and the rest of the platelets did not. This small percentage 2.28-positive platelets may reflect a small number of nonresting platelets present in the circulation in normal subjects. These platelets may have undergone the secretion reaction and may be responsible for the basic levels of platelet-specific proteins like β -thromboglobulin in plasma. On the other hand, we also observed a heterogeneity in platelets treated with thrombin in vitro. Some platelets did not express the antigen on the surface after stimulation. Further studies must be carried out to characterize these subpopulations, and flow cytometric sorting will provide a means to these studies.

Five other antiplatelet MoAbs that react preferentially with activated platelets have been described recently. KC_4 ³⁶ and S_{12} ^{37,38} identify a platelet α -granule membrane protein of mol wt 140,000 that is expressed on the platelet plasma membrane during degranulation. This protein may be a component of the internal α -granule membrane that is fused with the plasma membrane during activation. An antibody described in preliminary form, P_7 ,³⁹ may be directed against the same protein. The number of binding sites on stimulated platelets of these antibodies, 13,400, 9,600, and 11,200 molecules per platelet respectively, is in the same range as our antibody 2.28: 12,600 molecules per platelets. The other characteristics of 2.28, however, indicate that 2.28 identifies a different protein. Two antibodies whose binding to platelets is not dependent on platelet secretion recognize changes in the conformation or microenvironment of the membrane glycoprotein IIb-IIIa complex. Antibody 7E3⁴⁰ binds both to resting and activated platelets; the rate of binding, however, is increased after platelet activation. PAC1⁴¹ recognizes an epitope on the IIb-IIIa complex that is exposed after platelet activation.

Interest in the role and detection of activated platelets is mainly based on the hypothesis that activated platelets may have an important role in arterial disease and in sudden clinical events such as myocardial infarction and stroke that accompany it. Many tests of platelet function have been devised to test this hypothesis; a direct test for the detection

of activated platelets in the circulation, however, is lacking thus far. MoAbs specific for activated platelets are of potential value in this aspect.

We used flow cytofluorometric analysis to test the hypothesis that activated platelets *in vivo* may be detected with antibody 2.28. Cardiopulmonary bypass was chosen as a model since it has been shown that platelet activation occurs during this procedure and that afterwards degranulated platelets are present in the circulation.⁴²⁻⁴⁴ The number of platelets that expressed the 2.28 antigen on their surface was significantly increased after extracorporeal perfusion. There was a correlation with the β -thromboglobulin levels in plasma, and this supports the assumption that the number of 2.28-positive platelets represent platelets that are not in a resting state. We cannot exclude the possibility that some molecules on the platelets that are recognized by 2.28 are leukocyte derived. During cardiopulmonary bypass degranulation of these cells occurs,⁴⁵ and we observed 2.28 antigen localized in granules in monocytes (data not presented). A small increase in the number of 2.28-positive platelets was observed before extracorporeal perfusion in the patients as compared with the controls. This may have been caused by the fact that the first blood sample in the patients was taken via a catheter after the beginning of the operation, immediately before the extracorporeal perfusion.

Recently George et al⁴⁶ studied the binding of antibody

S12 to platelets in patients after cardiac surgery with cardiopulmonary bypass. In these patients they found no increased expression of the α -granule membrane protein GMP-140, the protein to which S12 binds. In contrast we found secretion of both α -granules and lysosomal granules in our patients. It seems unlikely that differences in the time of extracorporeal perfusion (average, 74 minutes v 92 minutes in our study), as suggested by George et al, explain the differences in secretion in these and other studies.⁴²⁻⁴⁶

To evaluate the usefulness of the test in thrombosis we studied two patients with acute deep venous thrombosis. Both had an increased number of 2.28-positive platelets in the circulation. Further studies in patients with clinical conditions that are known to be associated with a high risk of thromboembolic complications and studies on the effects of antithrombotic agents are needed to confirm the potency of our test. Once this test or a combination of platelet function tests and classic risk factors has been shown to be able to detect persons who will develop a myocardial infarction or stroke in the future, our ability to diagnose and treat patients with thrombotic vascular diseases will be enhanced.

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The Regulation of Human Factor V by a Neutrophil Protease

By Adrian M. Oates and Hatem H. Salem

Neutrophils activated with serum opsonized zymosan, soluble heat-aggregated IgG, and ionophore A23187 in the presence of calcium release a material capable of initially activating factor V. Subsequent inactivation of factor V was only observed with neutrophil releasate derived from IgG and ionophore. In this study we examine the nature of this neutrophil activity and investigate its role in the regulation of factor V/Va. From early in the fractionation it was apparent that the cells contained different enzymes capable of cleaving factor V. The most active of these was isolated and found to be an isomer of human neutrophil elastase. The purified protease caused a dose-dependent activation of isolated factor V to a maximum of threefold. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, single-chain factor V was cleaved to form intermediates of 100 and 91 kilodaltons (kD). Coagulant activity correlated with the formation of a 97-kD heavy and 77-kD

light chain. On prolonged incubation the formed factor Va(e) was inactivated in association with proteolysis of the 97-kD band to smaller peptides and cleavage of the 77-kD light chain to a molecular weight of 75 kD, which is similar to thrombin-activated factor Va light chain. Neutrophil elastase also caused rapid inactivation of thrombin-activated factor V, factor Va(t). These observations suggest that elastase cleaves factor V at sites distinct from that by thrombin and therefore represents a novel factor V activation pattern. It is proposed that upon neutrophil activation elastase is secreted into the plasma milieu to initiate factor V activation. This serves to generate small amounts of thrombin that, in turn, by positive feedback fully activates factor V and thus amplifies the coagulation reaction.

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FACTOR V is a single-chain polypeptide that circulates in plasma as a zymogen. Once activated to factor Va it acts as an essential cofactor for the enzyme factor Xa in the conversion of prothrombin to thrombin.¹ Thrombin is the major activator of factor V cleaving the molecule into a heavy chain, a light chain, and an activation peptide.² Both the heavy and light chains of factor Va are essential for the expression of procoagulant activity.³ In addition, the light chain acts as a cofactor for thrombin by accelerating its ability to activate protein C 50-fold.⁴

Factor V is also activated by a platelet protease.⁵ This protease is, however, cytosolic in location and not secreted during platelet activation, which suggests that it is unlikely to participate in the physiological regulation of hemostasis.

Bovine endothelial cells have also been shown to contain a homocysteine-dependent protease that activates factor V.⁶ This observation may account for the increased incidence of thrombosis in patients with homocysteinuria.

Recently, it has been demonstrated that the prothrombinase complex can assemble on the surface of neutrophils.⁷ This raises the possibility that these cells may play a role in the regulation of factor V activity.

In preliminary experiments, we have observed that neutrophils activated with serum opsonized zymosan, soluble heat-aggregated IgG, and ionophore A23187 in the presence of

calcium released an enzyme(s) capable of initially activating and subsequently inactivating factor V.

In this paper we examine the nature of this neutrophil activity and its possible physiological relevance.

MATERIALS AND METHODS

All materials were purchased from Sigma Chemical Co (St Louis) except for platelin (General Diagnostic—Warner Lambert Co, Morris Plains, NJ); chromogenic substrates S2484, S2238, and S2222 (Kabitrum, Stockholm); heparin (Weddel Pharmaceuticals, Sydney, Australia); ¹²⁵I-Na (Amersham Corp, Sydney, Australia); iodoigen and sodium dodecyl sulphate (SDS) (Pierce Chemical Co, Rockford, IL); NaCl and EDTA (Ajax Chemical Co, Sydney Australia); Ultrogel AcA 34 (LKB, Bromma, Sweden); and SP-Sephadex C50 and Sepharose 6B CL (Pharmacia Fine Chemicals, Uppsala, Sweden).

Coagulation factors were all of human origin. Factor V,⁸ factor X and prothrombin,⁹ thrombin,¹⁰ and fibrinogen¹¹ were purified and/or activated as indicated. A sheep antibody directed against human neutrophil elastase was purchased from ICN Immunobiologicals, Lisle, IL.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli.¹² Protein assays were performed by the Bradford method¹³ using the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA). Factor V coagulant activity was measured in a one-stage assay using a purified component system.¹⁰ Factor V was radiolabeled with ¹²⁵I-Na by using the iodoigen method (Pierce). The specific activity of the labeled material ranged from 1,000 to 1,500 cpm/ng.

Neutrophils were isolated by Ficoll-Hypaque sedimentation¹⁴ and were shown to consist of 97% neutrophils, 2% monocytes, and 1% lymphocytes as assessed by differential staining. Activation of neutrophils was performed by incubating 4×10^5 neutrophils with 5 µg/mL soluble heat-aggregated IgG, 0.5 µg/mL serum opsonized zymosan, or ionophore A23187 (2.5 µmol/L) in the presence of 1.25 mmol/L CaCl₂ in a total volume of 200 µL at 37°C for 20 minutes in a Patons Aggregometer (Scarborough, Canada) with stirring. The cells were then removed by centrifugation at 10,000 g for 30 seconds and the releasate tested for its effects on factor V clotting activity. Hirudin (1 U/mL) was included to prevent any thrombin-induced activation of factor V.

Soluble heat-aggregated IgG was prepared from a 2% solution of Cohn fraction II (Sigma) in 0.15 mol/L NaCl. Two hundred

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milligrams of IgG was heated for ten minutes at 63°C followed by rapid cooling to 0°C. Ammonium sulphate was added to a final concentration of 0.62 mol/L at 4°C for 60 minutes followed by centrifugation at 2,000 g for ten minutes at 4°C. The precipitate was then dissolved in distilled water and dialyzed against 0.15 mol/L NaCl overnight at 4°C.

Serum opsonized zymosan was prepared from a modification of the method by Brentwood and Henson.¹⁵ Zymosan (10 mg) suspended in 0.15 mol/L NaCl was boiled for 30 minutes in a glass tube. The mixture was allowed to cool and incubated with an equal volume of normal human serum for 30 minutes at 37°C to fix complement. The serum opsonized zymosan was then washed extensively with 0.15 mol/L NaCl to remove any unbound serum proteins.

For purification of the major factor V activator/inactivator from neutrophils, all purification steps were performed at 4°C and the buffers adjusted to contain 10 mmol/L benzamidine and 2 mmol/L EDTA unless otherwise stated.

The enzyme of interest was followed throughout the purification by examining the ability of test fractions to cleave ¹²⁵I-factor V as assessed on reduced SDS-PAGE with 4% stacking and 7.5% running gels. In this assay 1 U/mL ¹²⁵I-factor V was incubated with 10 µL of test fraction in a total volume of 100 µL for 20 minutes at 37°C. The buffer used was 20 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 5 mmol/L CaCl₂, and 5 mg/mL bovine serum albumin. We concentrated our efforts on the most potent enzyme that at low concentrations cleaved factor V to form peptides similar in molecular weight to those seen in Fig 4, whereas at higher concentrations of the enzyme only peptides migrating with dye front were observed. Only fractions demonstrating complete cleavage of single-chain factor V were pooled as indicated in Figs 2A to C.

Neutrophils were collected from a patient with chronic myeloid leukemia undergoing therapeutic leukapheresis and were shown to be 98% pure with respect to neutrophils by a differential cell count. The cells were washed three times in phosphate-buffered saline, pH 7.4, followed by four cycles of freeze-thawing. The cells were then extracted for 60 minutes by using 1% Triton X-100 and the cellular debris removed by centrifugation at 100,000 g for 30 minutes.

SP-Sephadex chromatography. The supernatant from the previous step was adjusted to pH 7.4 by using 1 mol/L Tris, pH 8, and applied to a SP-Sephadex C50 column (5 × 20 cm) equilibrated in 20 mmol/L Tris-base, pH 7.4, and 200 mmol/L NaCl at a flow of 100 mL/h. Unbound protein was washed through with five-column volumes of starting buffer. The column was then developed by using a 2-L total volume of 200 to 600 mmol/L NaCl linear gradient under the same conditions.

Heparin agarose chromatography. Fractions containing the protease of interest were pooled and concentrated by ultrafiltration using a YM-10 membrane (Amicon Corp, Lexington, MA). The concentrated material was adjusted to 200 mmol/L NaCl by the addition of distilled H₂O and applied to a heparin-Sepharose 6B CL affinity column (2.5 × 20 cm) equilibrated in 20 mmol/L Tris buffer, pH 7.4, containing 200 mmol/L NaCl. Unbound protein was washed through with five-column volumes of the aforementioned buffer followed by a linear 200 to 700 mmol/L NaCl gradient of 500-mL total volume. The flow rate was 50 mL/h and 5-mL fractions were collected.

SP-Sephadex chromatography. Fractions demonstrating factor V cleaving activity were pooled, concentrated by ultrafiltration, and adjusted to a final NaCl content of 220 mmol/L with distilled H₂O. This material was applied to a second SP-Sephadex C50 column (1 × 15 cm) equilibrated in 20 mmol/L Tris buffer, pH 7.4, and 220 mmol/L NaCl at a flow rate of 11 mL/h. Bound material was eluted by using a 220 to 600 mmol/L NaCl gradient of 50-mL total volume. One-milliliter fractions were collected.

RESULTS

Neutrophils stimulated with serum opsonized zymosan, soluble heat-aggregated IgG, and the ionophore A23187 in the presence of calcium released a material that caused the activation of factor V (Fig 1). A rapid threefold increase in factor V clotting activity was observed after a two-minute incubation of the releasate resulting from the addition of ionophore to neutrophils. This was followed by progressive inactivation of the activated factor V. Serum opsonized zymosan caused the release of material from neutrophils that activated factor V to a similar degree as that by ionophore releasate; however, peak activity was achieved after ten minutes of incubation. Soluble heat-aggregated IgG-derived releasate caused a twofold activation of factor V within six minutes of incubation, which on further incubation resulted in the loss of procoagulant activity. The observed changes in factor V activity were associated with proteolysis of the single chain of factor V as assessed by PAGE (results not shown).

For isolation of the factor V activator/inactivator from neutrophils, the starting material consisted of neutrophils from a patient with chronic myeloid leukemia undergoing therapeutic leukapheresis. These cells cleaved factor V with a pattern identical to normal neutrophils. From the outset of the purification process it was apparent that the neutrophil contained several proteases that were capable of proteolyzing factor V as seen as multiple cleavage patterns on SDS-PAGE. In this study, the most active protease was isolated by a combination of ion-exchange chromatography using SP-Sephadex and affinity chromatography on heparin agarose as detailed in Materials and Methods and Figs 2A to C.

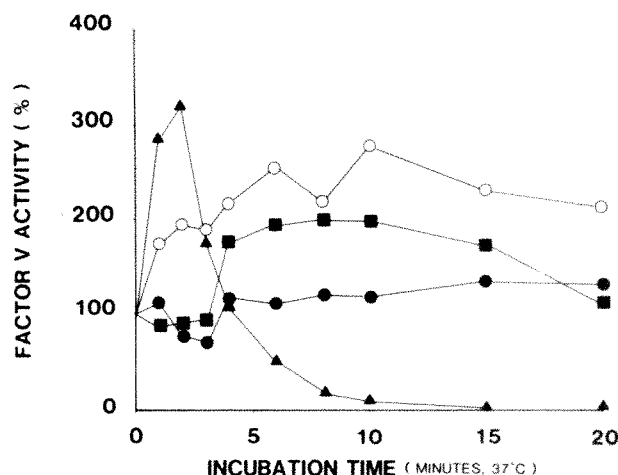


Fig 1. The effect of neutrophil releasate on factor V clotting activity. Neutrophils (4×10^5) were activated as detailed in Materials and Methods. After removal of the cells, 25 µL of the releasate was added to 1 U/mL factor V in a total volume of 50 µL and incubated at 37°C. The buffer used was 20 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 5 mmol/L CaCl₂, 0.1% gelatin, and hirudin (1 U/mL). Samples were removed at the times indicated and tested for factor V activity in a purified component clotting assay. ●—●, factor V plus neutrophil-alone releasate; ▲—▲, factor V plus ionophore-induced releasate; ○—○, factor V plus serum opsonized zymosan-induced releasate; ■—■, factor V plus heat-aggregated IgG-induced releasate.

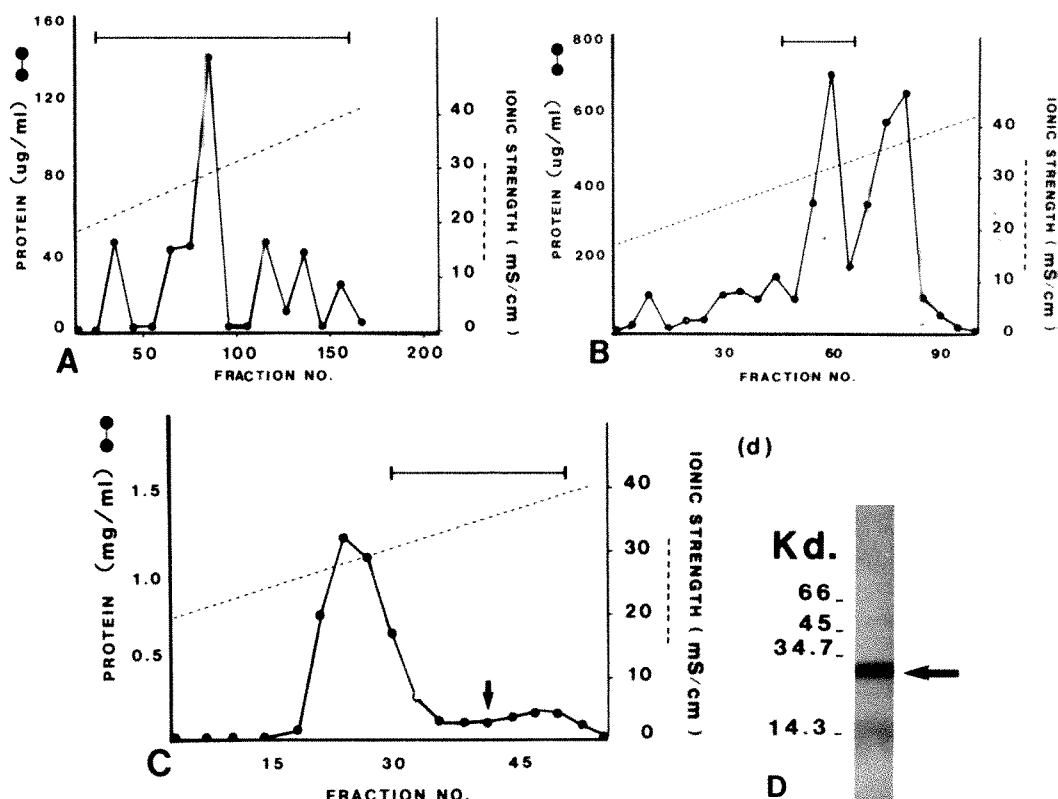


Fig 2. Isolation of the factor V activator from neutrophils. The enzymic activity was followed by the degree of cleavage of radiolabeled factor V on SDS-PAGE. The major enzyme was isolated by chromatography on (A) SP-Sephadex C50, (B) heparin-Sepharose 6B CL, and (C) SP-Sephadex C50 as detailed in Materials and Methods. Fractions were pooled as indicated (—). The final purified product corresponds to the arrow in C and is shown in D on 10% SDS-PAGE under nonreducing conditions stained with Coomassie brilliant blue R.

The final product shown in Fig 2D migrated as a tightly spaced doublet with an approximate molecular weight (mol wt) of 27 kilodaltons (kD) on unreduced SDS-PAGE and 29 kD under reducing conditions. The ability of the purified product to cleave factor V was inhibited by diisopropyl fluorophosphate (0.2 mmol/L), thereby suggesting that it is a serine protease. The material cleaved the elastase-specific synthetic substrates *N*-*t*-BOC-L-ALA *p*-nitrophenyl ester and S2484 at rates similar to those observed by Visser and Blout¹⁶ and Kramps¹⁷ for human neutrophil elastase, respectively. This ability was completely neutralized after its incubation with an equimolar concentration of α_1 -proteinase inhibitor. Furthermore, a commercial sheep antihuman neutrophil elastase antibody inhibited both the amidolytic and the ability of the enzyme to activate factor V. These observations suggest that the purified neutrophil protease is an isomer of elastase.

To confirm that the band shown in Fig 2D is the enzyme responsible for factor V cleavage, an SDS-PAGE gel containing the purified product was cut into 1-mm slices from which the protein was eluted and subsequently incubated with radiolabeled factor V. The only detectable cleaving activity corresponded to the major band shown in Fig 2D (results not shown).

Figure 3 shows the effect of purified neutrophil elastase on factor V clotting activity. The enzyme caused a concentra-

tion-dependent activation of factor V with a maximal three-fold increase in activity. SDS-PAGE analysis revealed that elastase initially cleaved factor V to form an intermediate consisting of a 100-kD heavy chain and a 91-kD light chain (Fig 4). On further incubation, these bands were proteolyzed to form a 97-kD heavy and 77-kD light chain designated factor Va(e). In other experiments, we documented that maximal coagulant activity correlated with the formation of the 97-kD and 77 kD peptides. Inactivation of factor Va(e) was associated with proteolysis of the 97-kD band to smaller peptides and the cleavage of the 77-kD band to a mol wt (75 kD) similar to that of thrombin-activated factor Va light chain.

These results contrast with factor V activation by thrombin, which results in a minimal tenfold enhancement in factor V activity. This more active form of factor V, herein referred to as factor Va(t), consists of a 112-kD heavy chain, a 75- to 71-kD light chain doublet, and a 150-kD activation peptide (Fig 5). Because elastase does not produce a major activation peptide that corresponds to that of thrombin-activated factor V, it is possible that this part of the factor V molecule is cleaved by the elastase.

This suggestion was examined by studying the effects of the addition of elastase to factor Va(t) as shown in Fig 5. Elastase caused rapid, progressive cleavage of the activation peptide into a band with a mol wt of 145 kD that on

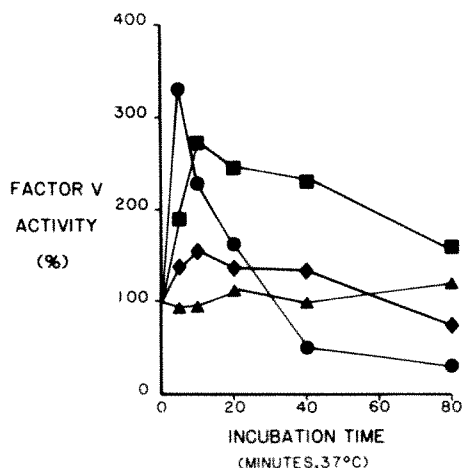


Fig 3. The effect of neutrophil elastase on factor V clotting activity. Factor V (1 U/mL) was incubated at 37°C with increasing concentrations of neutrophil elastase: Δ — Δ , 0 ng/mL; $\diamond$ — $\diamond$, 10 ng/mL; $\blacksquare$ — $\blacksquare$, 100 ng/mL; $\bullet$ — $\bullet$, 1 μ g/mL. The buffer used was 20 mmol/L Tris, pH 7.4, containing 150 mmol/L NaCl, 5 mmol/L CaCl₂, and 0.1% gelatin. At varying times subsamples were removed and tested for factor V activity in a purified component clotting assay.

continued incubation was cleaved to smaller mol wt peptides. The loss of coagulant activity was correlated with degradation of the heavy chain of the molecule. In contrast, the light chain undergoes minimal proteolysis, which indicates that there are no accessible elastase cleavage sites within this peptide. These observations confirm that elastase cleaves factor V at sites distinct from thrombin cleavage sites.

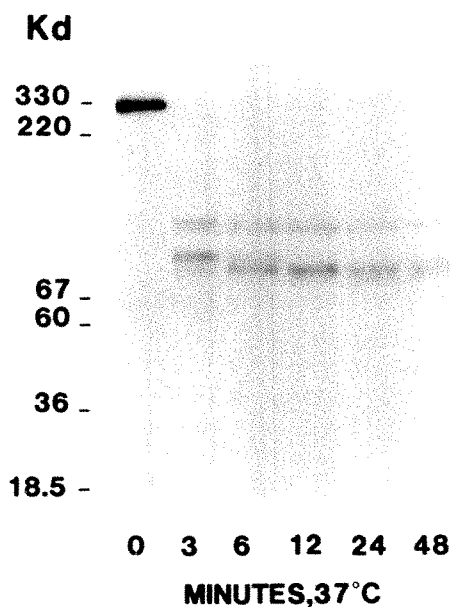


Fig 4. The cleavage pattern of ¹²⁵I-factor V by neutrophil elastase. ¹²⁵I-factor V (1 U/mL) was incubated at 37°C with 500 ng/mL neutrophil elastase. Subsamples were removed at the times indicated into reducing buffer, boiled, and run on SDS-PAGE with 4% stacking and 7.5% running gels. Gels were then dried and autoradiographed.

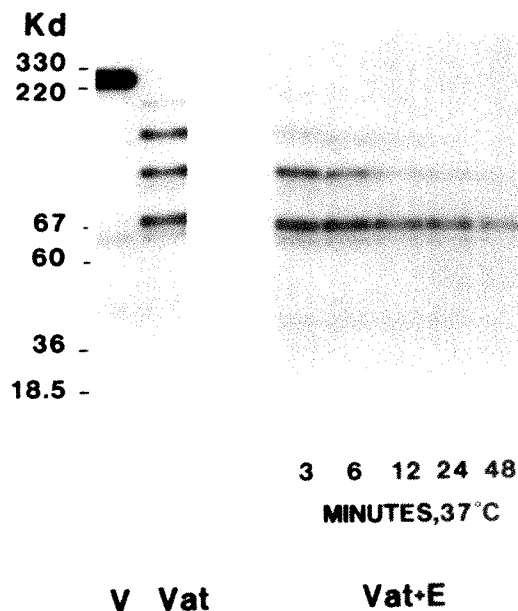


Fig 5. The effect of neutrophil elastase on thrombin-activated factor V. ¹²⁵I-factor V (1 U/mL) was incubated at 37°C with α -thrombin (1 U/mL) for 20 minutes, at which time hirudin (2 U/mL) was added and the incubation continued a further five minutes. Neutrophil elastase (250 ng/mL) was then added and subsamples removed at the indicated times for electrophoresis on 7.5% SDS-PAGE under reducing conditions followed by autoradiography. Factor V is represented by V, thrombin-activated factor V by Vat, and neutrophil elastase by E.

In an attempt to determine the physiological relevance of the effects of elastase on factor V we performed the following experiments. Normal whole blood was collected directly into a glass tube containing radiolabeled factor V, and the subsequent pattern of factor V cleavage during clot formation was followed on PAGE. The cleavage pattern of factor V resembled that produced by thrombin alone. We were unable to detect fragments similar to those produced by elastase. The activation peptide of factor Va(t) that is susceptible to elastase degradation remained stable over the course of the incubation. Similar patterns of factor V cleavage were observed when normal blood was substituted with either neutrophilic or neutropenic whole blood.

DISCUSSION

In the coagulation reaction, factor V acts as an essential cofactor to the serine protease factor Xa, which converts prothrombin to thrombin in the presence of a suitable surface.¹ The platelet has been demonstrated to be an effective surface on which components of the prothrombinase complex can be assembled,¹⁸ and more recently other cells have been shown to mediate the formation of this complex, including monocytes, lymphocytes, and neutrophils.⁷

Activation of factor V is necessary before it can participate in this reaction.² Thrombin is the most efficient physiological activator; it cleaves factor V to a molecule, factor Va(t), that is at least ten times more active than factor V in a coagulation assay. Since thrombin is the end product of the prothrombinase reaction, it is possible that other cellular or

circulating proteases can activate factor V earlier in the coagulation reaction. The small amounts of thrombin formed can then by positive feedback fully activate factor V and thus amplify the coagulation reaction.

Kane et al have described a platelet protease that activates factor V with a pattern distinct from that caused by thrombin.⁵ This activated form of factor V is at least fourfold more active than native factor V in a coagulation assay. However, the platelet protease is cytosolic in location and not released during platelet activation, which suggests that it is unlikely to play a major role in the physiological regulation of factor V activity.

Bovine aortic endothelial cells have also been shown to contain an activator of factor V, but this is expressed only in the presence of homocysteine.⁶ It is believed that this observation may account for the increased incidence of thrombosis in patients with homocystinuria. Normal fasting individuals have undetectable levels of homocysteine, thereby placing in doubt the physiological importance of this mechanism of factor V activation in normal hemostasis.

In this study, we have observed that the stimulation of human neutrophils with serum opsonized zymosan, soluble heat-aggregated IgG, and the ionophore A23187 in the presence of calcium is associated with the release of a molecule(s) that causes the activation of factor V. The purification of this activity was complicated by the fact that the neutrophils contained a number of proteases that are capable of cleaving factor V. We have concentrated on the most active of these molecules, and our results suggest that it is an elastase. The protease was neutralized by a sheep antihuman neutrophil elastase antibody and α_1 -proteinase inhibitor and inactivated by diisopropyl fluorophosphate thereby suggesting that it is a serine enzyme. It cleaved synthetic substrates that are specific for elastase. On SDS-PAGE the protease migrated as a tightly spaced doublet, with an approximate mol wt of 29 kD on reduced gels and 27 kD when run unreduced. The purified material therefore appears to correspond to the elastase isomer E4 described by Baugh and Travis.¹⁹

The ability of elastase to activate factor V was originally suggested by Schmidt et al.²⁰ These workers demonstrated a rapid activation of factor V by a range of elastase isomers. Their studies were performed with a one-stage clotting assay for the measurement of factor V. The amount of elastase used (4 μ g/mL) was far in excess of that released during normal whole blood clotting.²¹ Nevertheless, the initial activation was followed by a lengthening of the clotting time that corresponded to the inactivation of activated factor V. Egbring et al²² noted a twofold activation of the factor V molecule upon incubation of plasma with a mixture of elastase isomers at levels seen in the plasma of patients with septicemia²² or acute leukemia,^{22,23} whereas at higher levels (400 μ g/mL) only inactivation was observed.

We have demonstrated that the incubation of purified neutrophil elastase with factor V resulted in a concentration-dependent, two- to threefold activation followed by inactivation of the formed factor Va(e). A maximal increase in factor V activity correlated with the appearance of bands on

SDS-PAGE different from those produced by thrombin, the platelet protease described by Kane et al,⁵ or the homocysteine-dependent factor V activator⁶ and therefore represents a novel activation pattern.

The inactivation of the formed factor Va(e) was associated with cleavage of the 97-kD peptide that on continued incubation was degraded to smaller mol wt peptides. The 77-kD peptide was also processed to a molecule with a mol wt of 75 kD that is similar to the light chain of thrombin-cleaved factor V. This product appears to be slightly more resistant to subsequent degradation by the elastase, but with prolonged incubation it is cleaved to smaller peptides that migrated with the dye front. This part of factor Va(e) may, therefore, correspond to the factor Va(t) light chain in view of the mol wt similarity and resistance to elastase cleavage. This raises the possibility that the factor Va(e) light chain, like the factor Va(t) light chain, may also act as a cofactor to thrombin by enhancing its ability to activate protein C and thus amplify the natural anticoagulant system.⁴

Elastase has previously been shown to be released from the azurophilic granules of the neutrophil after incubation with serum opsonized zymosan, heat-aggregated IgG, and the calcium mobilizer A23187 in the presence of calcium.¹⁵ Therefore, our finding that the most potent protease contained within the neutrophil is an elastase is in keeping with the effects of neutrophil stimulation on factor V activity shown in Fig 1.

Our inability to demonstrate the presence of elastase-induced factor V cleavage products during whole blood clotting in vitro does not negate that this event could be physiologically important. Elastase may play a role in the activation of trace amounts of factor V very early in the coagulation reaction. The small amounts of thrombin generated may then by positive feedback fully activate factor V and thereby mask the cleavage pattern caused by elastase.

The potential of elastase to modulate factor V activity during whole blood clotting is dependent upon the ability of circulating inhibitors to inactivate the released protease. The most potent of these is the serpin, α_1 -proteinase inhibitor. When neutrophils undergo activation, oxygen metabolites are simultaneously released into the local environment with the elastase. These radicals serve to oxidize and inactivate the active site of the serpin. It has been estimated that 1.25×10^5 neutrophils have the potential to inactivate 162.5 to 325 μ g of α_1 -proteinase inhibitor via oxidative attack.²⁴ Thus, in inflammatory reactions the elastase released into the micro-environment could be protected from the inhibitory effects of α_1 -proteinase inhibitor and therefore act as an important modulator of the factor V activity and subsequent fibrin formation that characterize this pathological process.^{25,26} Furthermore, it is possible that the apparent factor V deficiency in patients with both acute²² and chronic myeloid leukemia²⁷ could be explained by the release of elastase under conditions of marked leukocytosis.

Although we have concentrated our studies on the effects of neutrophil elastase on factor V, our initial observations suggested that a number of neutrophil enzymes were capable of cleaving factor V. It would therefore be more appropriate

to suggest that neutrophils modulate factor V activity by the combined effect of these enzymes. The role of the other neutrophil enzymes in the regulation of factor V activity is the subject of our current studies.

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Regulation of Transferrin Receptor Expression in Myeloid Leukemia Cells

By Raymond Taetle, Steven Ralph, Shelley Smedsrud, and Ian Trowbridge

Surface transferrin (Tf) receptors are displayed by cultured human hematopoietic cells and provide Fe required for cell growth. Cell cycle status, cell density in culture, exposure to Fe, and differentiation alter Tf receptor display by myeloid leukemia cells. To investigate mechanisms controlling Tf receptor expression, rates of receptor synthesis and steady-state mRNA levels were measured in HL60 promyelocytic cells grown in serum and serum-free media or after differentiation in response to dimethylsulfoxide (DMSO). Although surface binding sites were unchanged during the first three days in culture with serum or in serum-free media containing Tf, by the third day, rates of receptor biosynthesis and steady-state mRNA levels declined, consistent with cell density-dependent, receptor regulation. Cells grown with soluble Fe instead of Tf showed reduced Tf binding sites, rates of receptor synthesis, and Tf receptor mRNA. When cells grown with Fe were subcultured, Tf receptor mRNA levels increased within 15 minutes and peaked by 24 hours. This was followed by a decline in receptor and gene expression so that by three days cells grown in the presence of Fe expressed approximately four times fewer receptors and had half the rates of Tf receptor synthesis and mRNA levels of cells grown in

serum or Tf. Cells treated with DMSO showed a rapid decline in surface receptors, receptor synthesis, and steady-state mRNA levels. Modulation of Tf receptor expression was not due to redistribution between the cell surface and an internal receptor pool. In each instance, concurrent assessment of *N-ras* transcripts showed that changes in Tf receptor mRNA levels were not due to generalized alterations in protein synthesis. Exposure of cells grown in Fe or treated with DMSO to cycloheximide did not alter Tf receptor mRNA levels, thereby suggesting that receptor expression was not regulated by posttranscriptional processes dependent on protein synthesis. Actinomycin D inhibition of Tf receptor mRNA was compatible with a transcript half-life of approximately 2.2 hours. Nuclear transcription studies showed reduced rates of Tf receptor transcription after culture with Fe or exposure to DMSO. The present studies show complex patterns of Tf receptor gene regulation in cultured myeloid leukemia cells and demonstrate that transcriptional regulation is a major mechanism controlling Tf receptor gene expression in response to Fe and differentiation.

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SURFACE TRANSFERRIN (Tf) receptors are displayed by cultured human hematopoietic cells, and their expression is modulated by a variety of regulatory mechanisms.¹⁻⁸ To characterize mechanisms regulating transferrin receptor expression on human leukemia cells, Tf receptor synthesis and steady-state mRNA levels were examined in HL60 promyelocytic leukemia cells grown in fetal bovine serum (FBS) and serum-free media⁴ or after differentiation in response to dimethylsulphoxide (DMSO).

Tf receptors on HL60 cells grown in serum-free medium in the presence of Fe⁴ increased within the first 24 hours of culture and reached levels similar to cells grown in serum or with human Tf. This was followed by a decline in receptor expression so that, by 48 hours, cells grown in the presence of Fe expressed approximately four times fewer receptors than

cells grown in serum or Tf.⁴ Steady-state Tf receptor mRNA levels and rates of receptor biosynthesis in HL60 cells grown in the presence of Fe showed corresponding changes. Similarly, decreased expression of Tf receptors after induction of HL60 cell differentiation was preceded by lower levels of Tf receptor mRNA and reduced receptor biosynthesis. Although cell surface Tf receptor expression by HL60 cells grown in serum or Tf remained constant during three days in culture, reduced levels of Tf receptor mRNA and receptor biosynthesis were found by day 3. Thus, differences in the surface Tf receptor expression by HL60 cells grown in the presence of Fe reflected the influence of Fe on the kinetics of cell density-dependent regulation of the receptor. Modulation of Tf receptor expression was not due to redistribution of receptors between the cell surface and an internal pool. Incubation of cells with cycloheximide in Fe, Tf, or with DMSO did not influence the steady-state levels of Tf receptor mRNA, thereby suggesting that receptor expression was not regulated by posttranscriptional processes dependent upon protein synthesis. Nuclear transcription assays confirmed that DMSO and Fe reduced rates of Tf receptor gene transcription. The present studies show that transcriptional regulation is a major mechanism controlling myeloid leukemia cell Tf receptor expression in response to Fe and differentiation.

METHODS

Cell cultures. HL60 cells were originally a gift from Dr Stephen Collins, University of Washington, Seattle, and have been maintained in RPMI 1640 medium with 10% FBS (JMI International, Kansas City, KA). Serum-free cultures were established from these cells and maintained for more than three years in RPMI 1640 medium supplemented with ethanolamine, 5 μ mol/L; insulin, 5 μ g/mL; selenium, 2.5 nmol/L; and either transferrin, 25 μ g/mL, or 5 μ mol/L Fe nitriloacetate (NTA).⁴

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For growth assays, cells were seeded at 2×10^5 /mL and viable cell counts performed daily using trypan blue dye exclusion. To induce granulocyte differentiation, cells grown with FBS were seeded at 3×10^5 /mL with 1% DMSO and viable cell counts performed daily.

Immunofluorescence staining. Staining with monoclonal, anti-Tf receptor antibody B3/25 was performed as previously described.^{4,9} A mixture of mouse antibodies served as a control. Positive cells were enumerated by using an Ortho 50H cytofluorograph (Ortho Diagnostics, Westwood, MA). DNA content was determined by propidium iodine staining and cell cycle status determined by a computer program as previously described.^{4,9} The percentage of cells in S phase was confirmed by immunofluorescent staining of incorporated bromodeoxyuridine (BrdU).⁹

Radioligand binding. Surface Tf binding was measured at 4°C and 37°C as described.¹⁰ Briefly, Fe-saturated Tf (Miles Pharmaceuticals, Naperville, IL) was radiolabeled in a 30-second incubation with 5 µg/mL chloramine-T to a specific activity of approximately 1.0×10^4 cpm/ng protein. The integrity of labeled material was confirmed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) and competition with unlabeled Tf.^{4,10} Cells grown under varying conditions were washed twice, incubated for two hours at 37°C in Tf-free medium, and then incubated with 100 nmol/L labeled Tf for two hours at 4°C. The cells were then washed and counted in a gamma counter. Nonspecific binding was determined by incubation with 200× unlabeled Tf.

The total cell-associated Tf was determined by incubation with labeled Tf at 37°C.¹⁰ Cells were incubated in Tf-free medium, labeled with Tf at 4°C as described earlier, and then incubated for periods of five minutes to 24 hours at 37°C. All three lines reached maximum binding within 30 minutes, and this time point was used for subsequent studies. Labeled cells were placed on ice and washed in phosphate-buffered saline (PBS) containing azide and the total cell-associated Tf determined by gamma counting.

Immunoprecipitation of metabolically labeled Tf receptors. To determine rates of Tf receptor synthesis, cells were metabolically labeled with ³⁵S-methionine (Amersham Corp, Arlington Heights, IL) and Tf receptors immunoprecipitated with B3/25. A total of 5×10^6 HL60 cells grown under varying conditions were washed, incubated for one hour in methionine-free medium, and resuspended in 1 mL of methionine-free medium containing 100 µCi ³⁵S-methionine. After four hours at 37°C, medium containing excess unlabeled methionine was added and the cells incubated for another hour. Labeled cells were washed three times in cold PBS and resuspended in 200 µL PBS with 10 µL 0.01 mol/L phenylmethylsulfonylfluoride.

To study cells grown in FBS or serum-free media, aliquots containing equal trichloroacetic acid (TCA) precipitable counts (5 to 10×10^6 cpm) were removed and incubated at 4°C for two hours on a mechanical rocker with 100 µL 10% Pansorbin suspension (Calbiochem-Behring, La Jolla, Ca) complexed to 28 µg rabbit antimouse immunoglobulin (Zymed Laboratories, S San Francisco) and 10 µg B3/25, monoclonal anti-Tf receptor antibody,² or an isotype-specific control. For studies of DMSO-treated cells, aliquots of cell lysates representing equal numbers of control and treated cells were analyzed. The immune complexes were pelleted in a microfuge, washed with PBS containing 0.3% NP-40, and layered over a 20% sucrose cushion. The tubes were spun for ten minutes in a microfuge and washed three times with buffer. The pellet was incubated for ten minutes with 0.5 mol/L LiCl and resuspended in buffer. The pelleted immune complexes were heated at 100°C for five minutes and electrophoresed on 7% or 10% polyacrylamide reducing (0.1% SDS) or nonreducing gels. Immunoprecipitated Tf receptors were scanned by digital matrix morphometry¹¹ through the courtesy of Dr William Neeley, Veterans Administration Medical Center, San Diego.

Extraction of cellular RNA. A quantity of 5×10^7 to 10^8 cells was washed in PBS and pelleted by centrifugation. While vortexing, cells were lysed with 2.3 mL 4 mol/L guanidine isothiocyanate containing 20 mmol/L Na acetate (pH 5.0) and 1 mol/L 2-mercaptoethanol. After 30 seconds, the cell suspension was immediately layered over 1.2 mL 5.7 mol/L CsCl/0.1 mol/L EDTA in a $7/16 \times 2\frac{1}{8}$ -in Beckman polyallomer tube (Beckman Instruments, Inc, Fullerton, CA). The lysed cells were centrifuged for 12 to 18 hours at 150,000 g at 20°C. The liquid was aspirated and inverted tubes drained for one minute. The bottom of the tube containing the RNA pellet was washed twice with 100 µL 7.5 mol/L guanidine HCl containing 25 mmol/L Na citrate, and 10 mmol/L 2-mercaptoethanol, twice resuspended in TE (10 mmol/L Tris/1 mmol/L EDTA) buffer, and added to 500 µL butanol:chloroform (1:4). After centrifugation, the supernatant was extracted twice more with butanol:chloroform and RNA precipitated with ethanol/Na acetate at -20°C for 12 hours. The RNA was reprecipitated by the addition of 1 mL cold 70% ethanol. This procedure was repeated and the pellet dissolved in TE buffer. RNA was quantitated spectrophotometrically at optical density 260.

Northern and slot-blot hybridization. For Northern blot analysis, cellular RNA (5 µg) was electrophoresed in 1% agarose gels containing formaldehyde¹² and the gel washed twice for 30 minutes with 500 mL deionized water and once with 20 mmol/L Na phosphate, pH 6.5. RNA was transferred onto nylon membranes presoaked in 20 mmol/L Na phosphate.¹³ Prehybridization and hybridization were performed as described by others.¹⁴ The probes used were a 2.96 kilobase (kb) *Hind*III fragment from the human transferrin receptor cDNA clone, pCD-TR1¹⁵ (kindly provided by Dr Lucas Kuhn) or a 1.5-kb p52C-*Eco*RI fragment of the HL60 *N-ras* gene purified from a reconstructed pBR322 plasmid¹⁶ (kindly provided by Dr Inder Verma). *N-ras* was chosen as a control because previous studies showed little change in expression of this gene during HL60 differentiation.¹⁷ Probes were labeled by nick translation according to the manufacturer's instructions (Nick Translation Kit, Bethesda Research Laboratories [BRL], Bethesda, MD). The filters were washed in 1.5 mmol/L Na citrate, pH 7, 15 mmol/L NaCl, and 0.1% SDS at 52°C. In some studies, the same Northern blot was "stripped" and probed with the *N-ras* insert.

Slot-blot analysis was used to compare Tf receptor or *N-ras* RNA content. All samples analyzed were previously shown by Northern blot to contain a single 4.9-kb transferrin receptor transcript without evidence of degradation. (For an example, see Fig 5.) Biotrans nylon membranes (ICN Biomedicals, Irvine, CA) were presoaked in water and then 10× sodium citrate/sodium chloride (SSC) and placed in a slot-blot apparatus (Schleicher and Schuell, Keene, NH). RNA was denatured with a solution of 6.15 mol/L formaldehyde and 10× SSC at 65°C for 15 minutes. Serial twofold dilutions beginning with 10 µg RNA were loaded and the wells washed with 10× SSC. The filters were dried in a vacuum oven and hybridized to the Tf receptor probe as described earlier. The same filters were then placed in 10 mmol/L Na phosphate, pH 6.5, and 50% formamide and incubated at 65°C for one hour to remove the Tf receptor probe. The membrane was washed in 2× SSC and 0.1% SDS at room temperature and prehybridized as described before. The membranes were then hybridized to the *N-ras* probe.

Determination of cellular ferritin. Cellular ferritin was quantified as previously described.⁴ Briefly, 10^7 HL60 cells grown for varying periods in ethanolamine, insulin, selenium, and ferric nitrilotriacetate (EIS-Fe) medium were lysed by freezing and thawing on acetone/dry ice. Complete lysis was confirmed by visual examination. Cell-free supernatant was recovered by ultracentrifugation, diluted to 2 mL, and assayed for ferritin by radioimmunoassay according to the manufacturer's instructions (Becton Dickinson, Orangeburg, NY).

Effects of cycloheximide and actinomycin D on transferrin receptor mRNA. To determine the effects of inhibiting protein or RNA synthesis on Tf receptor gene expression, HL60 cells were grown for 48 hours in FBS containing 1.0% DMSO or for 72 hours in ethanolamine, transferrin, insulin, and selenium (ETIS) or EIS-Fe. Cells were then incubated with media, 10 μ g/mL cycloheximide (Sigma Chemical Co, St Louis) for 2.5 hours, or 5 μ g/mL actinomycin D (Sigma) for two hours and washed and cellular RNA extracted.

Nuclear transcription assays. Transcription of nuclear mRNA was compared in cells cultured for 72 hours in FBS or EIS-Fe and in cells induced to differentiate with DMSO. The assays were performed as described by others.¹⁸ Briefly, 2 to 5 $\times 10^7$ nuclei were obtained by lysis in STKM buffer (30% sucrose, 40 mmol/L Tris, 37 mmol/L KCl, 12 mmol/L MgCl₂) with 0.5% NP-40. Extension of nuclear transcripts was carried out in a ten-minute incubation with 30 μ g/mL DNase (BRL) in the presence of 1 mmol/L CaCl₂ with 0.8 mCi/mL ³²P-guanosinetriphosphate (3000 Ci/mmol/L), 0.5 mmol/L adenosine triphosphate, 0.25 mmol/L uridine triphosphate, and 0.25 mmol/L cytidine triphosphate, followed by a 30-minute treatment at 45°C. The RNA was purified free of DNA, protein, and unincorporated isotope and precipitated with 10% TCA (4°C for 30 minutes) and the labeled RNA recovered on type HA nitrocellulose filters (0.45- μ m pore size). The filters were incubated with DNase I with 50 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, and 1 mmol/L CaCl₂ and the RNA eluted with 5 mmol/L EDTA/1% SDS at 68°C. The eluate was treated with proteinase K, extracted with phenol/chloroform, and precipitated at least twice in ethanol. Filters containing immobilized Tf receptor cDNA or pBR322 plasmid control DNA were prehybridized in 4 $\times$ Denhardt's, 50% formamide, and 1 mg/mL phenol/chloroform-extracted tRNA (Boehringer-Mannheim, Indianapolis) at 42°C for three hours. Hybridization was carried out as described.¹⁸ Hybridization to control wells containing no DNA was also performed in each experiment and showed no specific binding. Equal radioactive counts derived from each experimental treatment, including DMSO exposure, were loaded into each well. After hybridization for three days at 42°C, the filters were washed twice in 300 mmol/L NaCl, 2 mmol/L EDTA, and 10 mmol/L Tris, followed by a wash in 10 mmol/L NaCl, 2 mmol/L EDTA, and 5 mmol/L Tris. The filters were then incubated with 10 μ g/mL RNase A and 1 μ g/mL RNase T1 and autoradiographed. Incubation of nuclei with 2 μ g/mL α -amanitin completely abrogated new Tf receptor transcription.

RESULTS

In previous studies, we showed that log-phase cultures of HL60 cells grown in FBS- or TF-supplemented medium (ETIS) had similar numbers of Tf receptors, but whether measured by Tf binding or staining with monoclonal antibody B3/25, cells grown with soluble Fe (EIS-Fe) had substantially fewer receptors.⁴ As shown in Table 1, more extensive studies showed that this difference results from a marked temporal variation in Tf receptor expression on HL60 cells grown in EIS-Fe that was not observed under other culture conditions. During the first three days in culture, Tf receptor expression remained unchanged on cells grown with FBS or ETIS, whereas during the first day in culture, cells grown with EIS-Fe showed a transient five- to tenfold increase in Tf receptor expression. As a consequence, after one day in culture, cells grown with EIS-Fe expressed Tf receptor numbers similar to cells grown in FBS or ETIS but showed a rapid decrease in receptor expression during the ensuing 48 hours.

This modulation in Tf receptor expression by cells grown in EIS-Fe was not directly related to cell density or growth rate. As shown in Table 1, for the three-day culture period studied, HL60 cell growth was identical under all three culture conditions, and doubling times ranged from 22 to 24 hours. Further, cell cycle distributions were similar. As determined by propidium iodine staining, the proportion of cells in G₁ phase of the cell cycle ranged from 49% $\pm$ 4% to 53% $\pm$ 5% (mean $\pm$ SE, three to five determinations). In three independent experiments, the fraction of cells in S phase was determined by using BrdU incorporation. Consistent with data obtained by propidium iodine staining, cells grown in ETIS and EIS-Fe had similar relative proportions of cells synthesizing DNA (48% $\pm$ 5% and 44% $\pm$ 10% respectively).

To determine whether changes in Tf receptor expression on cells grown with EIS-Fe represented a redistribution between internal and cell surface pools,¹⁹ the total cell-associated Tf was measured at 37°C.⁴ As shown in Table 1, changes in the total receptor pool assessed by binding at 37°C closely paralleled changes in cell surface receptors, and relative distributions between surface and intracellular compartments remained constant. Thus, changes in surface Tf binding by cells grown with EIS-Fe were not the result of receptor mobilization from a preexisting, intracellular receptor pool.

To further characterize Tf receptor regulation in HL60 cells grown with FBS, ETIS, or EIS-Fe, steady-state Tf receptor mRNA and rates of receptor biosynthesis were investigated. Transferrin receptor mRNA levels were quan-

Table 1. Transferrin Binding at Various Cell Densities of HL60 Cell Lines

	Time (d)			
	0	1	2	3
FBS				
Cell counts	—	4.3*	7	14.0
Transferrin bound, -4°C	—	18.4†	10.8	16.8
Transferrin bound, -37°C	—	32.5	35	30.5
Transferrin receptor-positive (%)	—	90‡	89	93
ETIS				
Cell counts	—	4.0	6.8	14.0
Transferrin bound, -4°C	—	11.6	18.7	18.5
Transferrin bound, -37°C	—	35.0	33.5	36.0
Transferrin receptor-positive (%)	—	92	88	89
EIS-Fe				
Cell counts	—	4.6	8	15.0
Transferrin bound, -4°C	—	10.0	3.7	1.6
Transferrin bound, -37°C	—	33.4	8.7	7.1
Transferrin receptor-positive (%)	—	89	74	42
DMSO				
Cell counts	3.0	7.4	12.8	12.5
Transferrin bound, -4°C	13.5	1.7	1.1	1.3
Transferrin bound, 37°C	34	8.6	4.5	5.3

Cell cultures without DMSO were initially plated (time zero) at 2 $\times 10^5$ /mL.

* $\times 10^{-5}$ /mL.

† Nanograms bound/10⁶ cells.

‡ B3/25-positive cells by immunofluorescence.

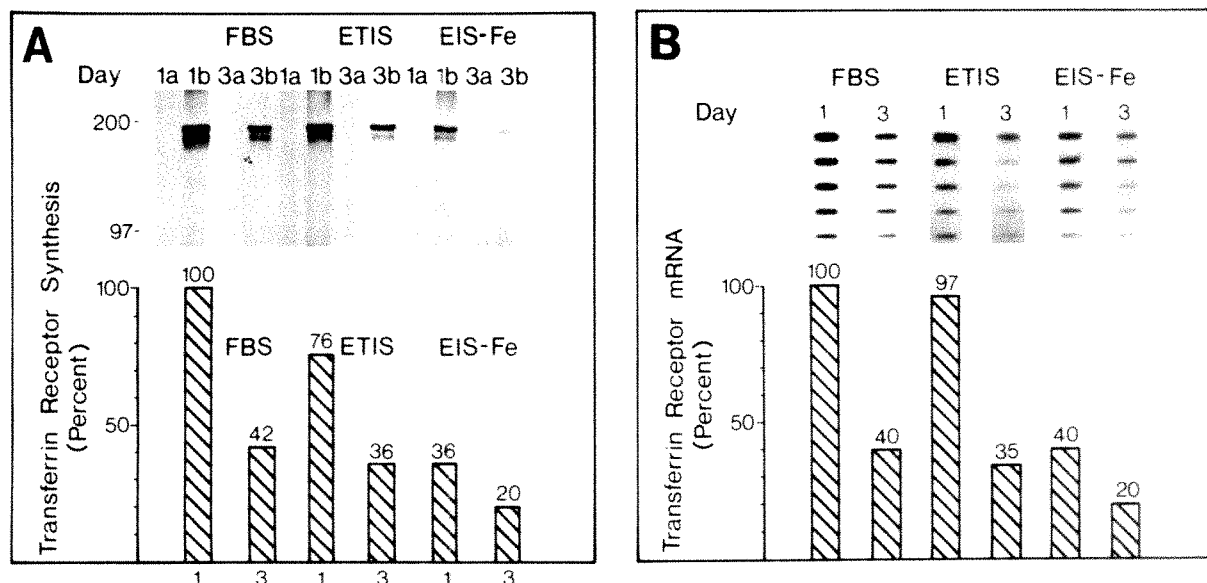


Fig 1. (A) One of three representative studies showing immunoprecipitation of ^{35}S -methionine-labeled transferrin receptors from HL60 cells grown for one or three days in FBS, ETIS, or EIS-Fe. Cells were labeled for four hours and aliquots containing equal TCA-precipitable counts immunoprecipitated with monoclonal, antitransferrin receptor antibody B3/25 (b lanes) or an isotype control (a lanes). Relative receptor synthesis was estimated by scanning nonreduced gels. (B) Total cellular RNA was extracted from cells after one and three days in culture and hybridized to a transferrin receptor, cDNA probe. Relative transferrin receptor RNA levels were estimated by scanning slot blots.

titated by slot-blot analysis of total cellular mRNA. When hybridized to the human Tf receptor cDNA probe on Northern blots, RNA preparations used in these studies showed a single 4.9-kb band. The rate of Tf receptor biosynthesis was estimated by pulse labeling with ^{35}S -methionine for four hours and analyzing immunoprecipitates by using SDS-PAGE under nonreducing conditions. As shown in Fig 1, the rates of receptor synthesis correlated well with steady-state levels of Tf receptor mRNA. After one day in culture, mRNA levels and receptor synthesis by cells grown in EIS-Fe were about one third that of cells grown in ETIS or FBS and declined even further after three days in culture. A twofold decrease was also observed in Tf receptor mRNA levels and rates of synthesis in cells grown in ETIS and FBS for three days. This suggests that, in cultures with FBS or Tf, cell density-dependent downregulation^{20,21} had been initiated through reduced Tf receptor biosynthesis, but because of the relatively long half-life of the Tf receptor glycoprotein,²² was not yet reflected by cell surface receptor expression.

Although these data were consistent with previous studies showing reduced Tf receptor expression by HL60 cells grown in EIS-Fe,⁴ they did not account for the transient elevation in Tf receptor levels detected in these cells after one day in culture. To address this point, Tf binding sites, Tf receptor synthesis, and mRNA levels at various times during the first 24 hours of culture in EIS-Fe were determined. A reproducible, rapid increase in Tf binding sites was observed during the first few hours, which increased to maximum levels within 24 hours (Fig 2). Although the precise time at which maximal mRNA and receptor synthesis occurred varied from experiment to experiment, Tf receptor synthesis and steady-state mRNA levels also consistently increased within 30 minutes after subculture and peaked by 24 hours (Fig 3A

and B). In contrast, no change was observed in *N-ras* transcripts. Despite the fact that Tf receptor numbers on cells grown in ETIS remained relatively constant during the three-day culture period, an increase in Tf receptor mRNA levels was also observed in these cells during the first 24 hours after subculture (Fig 3B).

When HL60 cells are cultured in EIS-Fe, reduced Tf receptor levels are accompanied by a tenfold increase in intracellular ferritin.^{4,7} To determine whether the transient increase in Tf receptors during the first day in culture was accompanied by reciprocal changes in ferritin, ferritin levels were determined at various times during the first 24 hours of

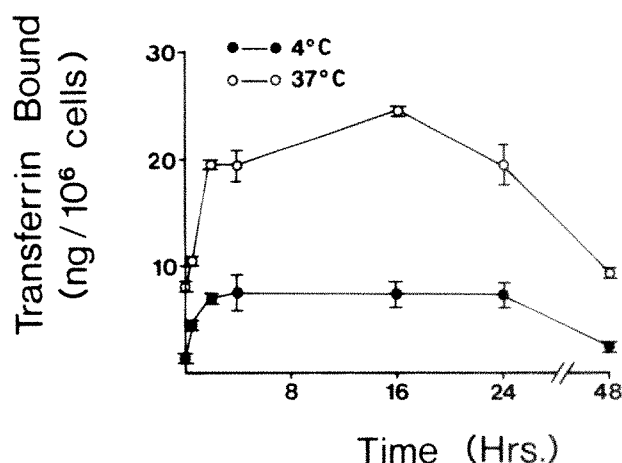


Fig 2. During the first 24 hours in culture, cells grown in EIS-Fe showed a rapid increase in surface and total cellular receptors. By 48 hours, surface display decreased. Results represent the means $\pm$ SE of four studies.

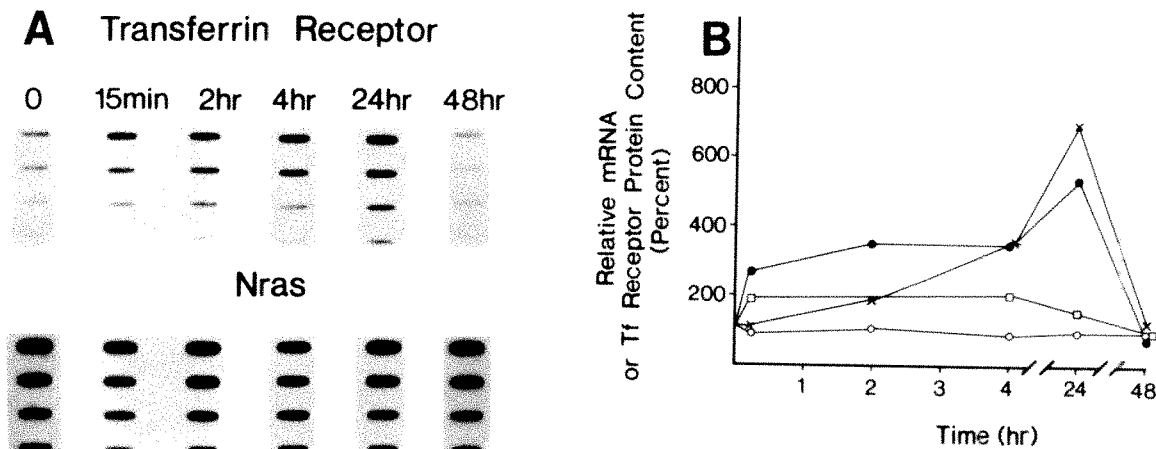


Fig 3. (A) Transferrin receptor mRNA levels increased rapidly in EIS-Fe-containing cells during the first 24 hours. At the times shown, total cellular mRNA was extracted and hybridized to a transferrin receptor or *N-ras* probe. Representative results from one of three studies are shown. (B) By scanning densitometry, transferrin receptor mRNA levels (●) and metabolically labeled receptors (X) increased during the first day in culture with EIS-Fe. A similar smaller increase occurred in Tf receptor transcripts in HL60 cells cultured in ETIS (□). In contrast, *N-ras* transcripts in EIS-Fe (○) were unchanged. Transferrin receptor transcripts and protein synthesis increased with varying kinetics from study to study.

culture with EIS-Fe. Values were similar to those previously reported^{4,7} (450 to 900 ng/10⁷ cells) and showed no change during the first day in culture. HL60 cells were also resuspended for 24 hours in "spent" three-day EIS-Fe medium that had been dialyzed against fresh medium by using tubing with a 3,000-dalton exclusion limit. Increased Tf receptors were again noted. Thus, the transient increase in Tf receptor expression by HL60 cells grown in EIS-Fe was not accompanied by reduced cell ferritin, nor was it influenced by macromolecules in HL60 conditioned medium.

Downregulation of Tf receptors also occurs when HL60 cells grown in FBS are induced to differentiate into granulocytes with DMSO² (Table 1). As shown in Fig 4, when HL60 cells were exposed to DMSO, within one day Tf receptor biosynthesis decreased by 90% and was accompanied by a similar decrease in steady-state levels of Tf receptor mRNA. In contrast, during the first two days, *N-ras* transcripts increased after DMSO treatment (Fig 4A). Thus, the early decrease in Tf receptor synthesis was specific and occurred before a generalized decline in protein synthesis seen on day

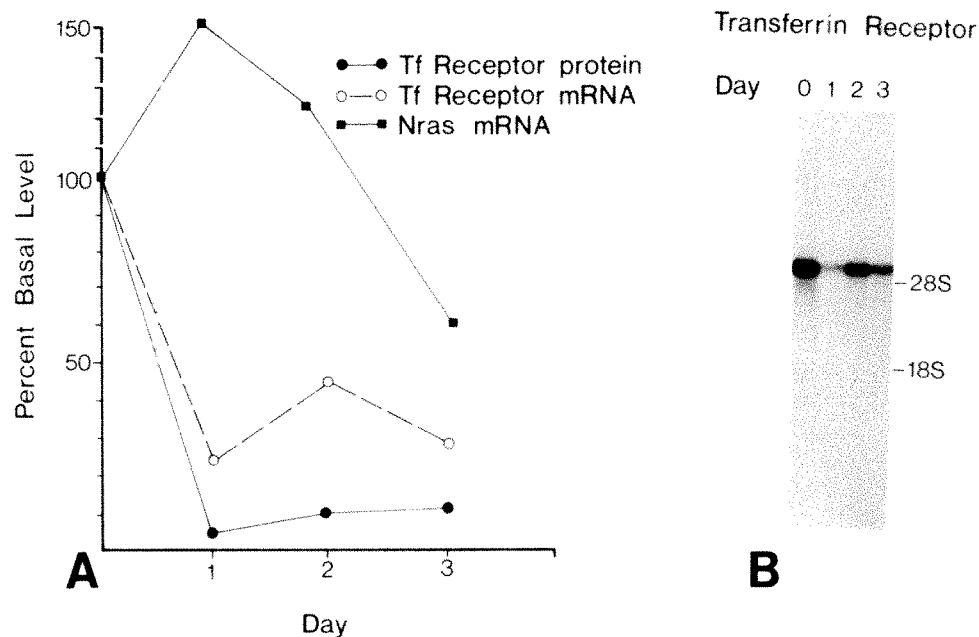


Fig 4. (A) Relative levels of metabolically unlabeled transferrin receptors, transferrin mRNA, and *N-ras* mRNA in HL60 cells cultured with 1% DMSO. Labeled receptor protein, receptor mRNA, and *N-ras* mRNA were quantitated by scanning gels or slot blots. Representative results from one of three studies are shown. (B) Total cellular RNA extracted from HL60 cells cultured for one to three days with DMSO and analyzed by Northern blotting. The decrease in transferrin receptor mRNA after one day is apparent.

3 that was reflected by decreased incorporation of ^{35}S -methionine into TCA-precipitable material and a reduction in *N-ras* transcripts.

Recent studies indicate that expression of some eukaryotic genes such as *c-myc*²³⁻²⁶ is regulated by posttranscriptional mRNA stabilization. When cells are cultured with protein synthesis inhibitors such as cycloheximide, the rate of mRNA degradation decreases, and steady-state mRNA levels increase.²³ To investigate whether similar mechanisms regulate Tf receptor expression, HL60 cells were incubated for two hours with cycloheximide and Tf receptor mRNA levels analyzed by Northern (not shown) and slot-blot hybridization. After cycloheximide treatment, cells cultured in EIS-Fe or ETIS for three days or incubated with DMSO for two days contained slightly decreased Tf receptor transcripts (Fig 5). In contrast, after incubation for two hours with actinomycin D, which inhibits RNA synthesis, Tf receptor transcripts decreased by 50% to 60% (Fig 5). The latter finding is compatible with a transcript half-life of approximately 2.2 hours. Thus, there was no evidence for Tf receptor transcript stabilization after inhibition of protein synthesis.

To confirm that Tf receptor gene regulation occurred at the level of gene transcription, nuclear transcription of Tf receptor mRNA was compared in cells cultured in FBS and Fe for 72 hours or induced to differentiate with DMSO. As shown in Fig 6A, cells grown with Fe showed rates of Tf receptor transcription approximately 1/5 that of cells grown in FBS. Similarly, Tf receptor transcription was three- to tenfold less in cells treated with DMSO for 24 or 48 hours than in control cells (Fig 6B). Because equal numbers of radiolabeled mRNA counts from each experimental condition were hybridized to the Tf receptor probe, these results may underestimate the actual decrease in Tf receptor gene transcription. However, these results confirm that Tf recep-

tor gene expression in response to Fe or DMSO is controlled at the level of gene transcription.

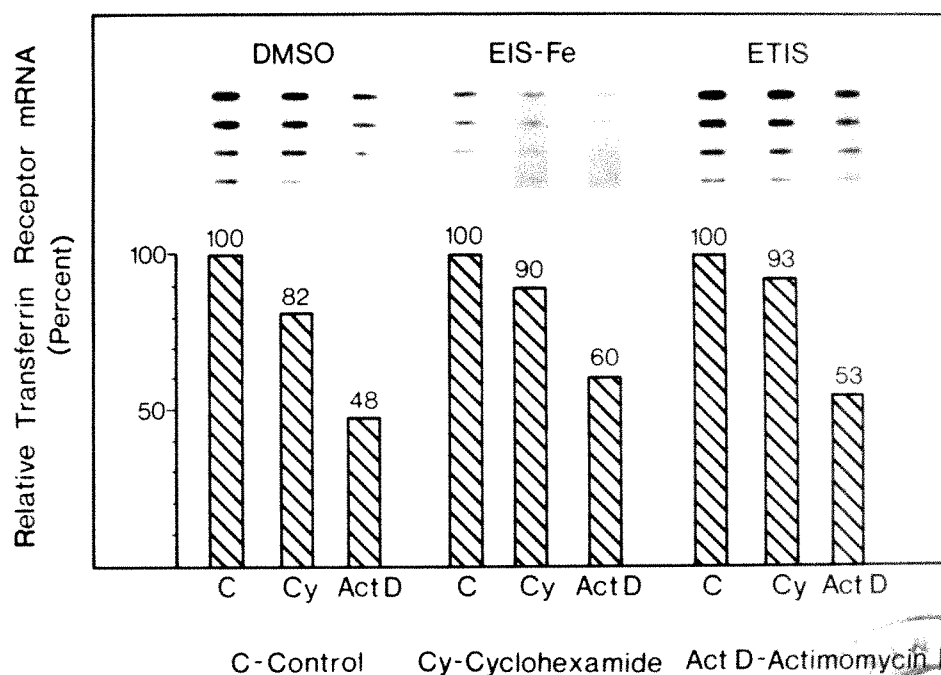
DISCUSSION

Tf receptor expression is closely coupled to important events in hematopoietic cell growth such as the transition of resting cells (G_0) to the DNA synthesis (S) phase of the cell cycle⁵ and induction of terminal myeloid leukemia cell differentiation.² Receptor expression is required for progress through the cell cycle⁵ and, in leukemia cell differentiation, is suppressed before detection of other differentiation programs.⁸ Therefore, mechanisms controlling Tf receptor gene expression are intimately involved in regulation of hematopoietic cell growth and differentiation.

The use of stable, variant HL60 lines allowed us to examine steady-state Tf receptor mRNA in a variety of regulatory settings and to correlate transcript levels with receptor synthesis. To minimize effects of cell growth on Tf receptor display, we initially studied growing cells with identical proliferation rates and cell cycle distributions. Using these conditions and DMSO induction, we determined that altered surface Tf binding reflected changes in the total receptor pool.

In previous studies of HL60 and other hematopoietic cells, Tf receptor display declined with increased cell density in culture.^{6,20,21} In the present studies, cells were grown in FBS and ETIS for only three days, and no change was observed in Tf binding. However, Tf receptor protein synthesis and mRNA levels were reduced by three days, thus indicating the onset of decreased receptor synthesis. It is likely, therefore, that prolonged culture would lead to decreased levels of surface transferrin receptors as described by others. The basic pattern of the cell density-dependent regulation of transferrin receptor expression in cells cultured in EIS-Fe was similar to that of cells grown in FBS and ETIS. The

Fig 5. To determine whether transferrin receptor mRNA levels were regulated by posttranscriptional processes requiring protein synthesis, mRNA was extracted from HL60 cells treated with DMSO, cultured in EIS-Fe for three days, or grown for three days in ETIS and transferrin receptor mRNA quantitated by slot-blot analysis. C, control cells; Cy, cells treated for 2.5 hours with cycloheximide; ActD, cells treated for two hours with actinomycin D. Representative results from one of two studies are shown.



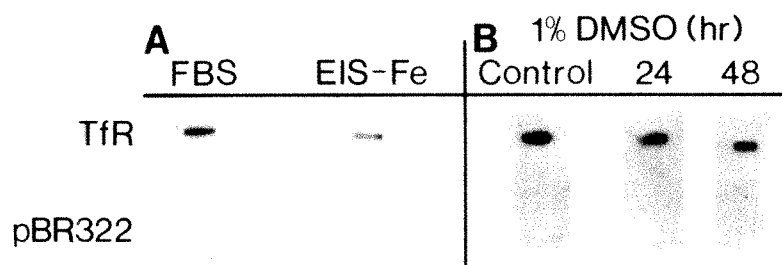


Fig 6. (A) RNA obtained by in vitro nuclear transcription from HL60 cells grown for 72 hours in FBS or in EIS-Fe was hybridized to the Tf receptor gene probe (TfR) or pBR322 plasmid control DNA. Scanning of autoradiographs showed fivefold greater transcripts from cells grown in FBS. One of two representative studies is shown. (B) Nuclear RNA from cells grown in FBS (control) or with 1% DMSO for 24 or 48 hours was hybridized to Tf receptor or plasmid control DNA. A three- to tenfold greater concentration of Tf receptor RNA was detected from control nuclei. Representative results from one of three studies are shown.

effect of Fe appears to be to trigger a more rapid onset of receptor downregulation at a time when the cells are still in exponential growth. In some eukaryotic cells, synthesis of Fe binding proteins is regulated by differential translational rates from "stored" mRNA.²⁷ Mammalian cells may also use similar "gene-sparing" mechanisms for gene regulation.²⁸ In present studies, coordinate changes in protein synthesis and Tf receptor mRNA ruled out this mechanism of gene regulation.

As expected based on previous work,^{3,4} cells grown with Fe showed reduced Tf binding sites. However, during the first 24 hours in culture, these cells rapidly increased receptor synthesis and mRNA production. This resulted in the transient expression of transferrin receptors at levels comparable to cells grown in FBS or ETIS. This increased receptor production was not accompanied by changes in cellular ferritin, nor was it due to secretion or depletion of macromolecules in the media. Thus, expression of transferrin receptors on cells cultured in EIS-Fe was influenced by two interacting in vitro regulatory mechanisms—cell culture density and exogenous Fe. The resulting pattern of Tf receptor display reflected enhanced expression induced by a lower culture density that is subsequently inhibited by Fe. Since *N-ras* transcripts showed no change, this regulation of Tf receptor synthesis was specific and not a consequence of general changes in protein production.

When HL60 cells were induced to differentiate by treatment with DMSO, surface Tf receptors decreased within one day.² This was the result of lower steady-state levels of transferrin receptor mRNA and a concomitant reduction in receptor synthesis, results consistent with a recent report by Ho et al.²⁹ Again, regulation of transferrin receptor gene activity was specific because *N-ras* transcript levels did not fall until a general reduction in protein synthesis occurred.

Some proliferation-related genes such as *c-myc* are regulated, at least in part, by posttranscriptional mRNA stabilization.²³⁻²⁶ When HL60 cells were incubated with cycloheximide, no changes in Tf receptor transcript levels occurred. Thus, regulation of the Tf receptor gene does not involve

posttranscriptional mechanisms requiring protein synthesis. Consistent with results recently reported by Rao et al,³⁰ nuclear transcription assays confirmed that cells grown with Fe had lower rates of Tf receptor gene transcription. After DMSO treatment, Tf receptor gene transcription was also reduced. This was observed despite the fact that equal radioactive counts were hybridized to each probe. Thus, the observed changes were not attributed to a generalized reduction in nuclear RNA synthesis.

Although cycloheximide did not alter Tf receptor gene expression, RNA synthesis was inhibited by actinomycin D. A two-hour pulse decreased Tf receptor transcript levels by slightly less than 50%, consistent with a transcript half-life of 2.2 hours. This figure is in close agreement with recent studies examining Fe effects on K562 Tf receptor gene transcription.³⁰

In vitro, Tf receptor gene expression is rapidly modulated and results obtained when using cells grown with Fe indicate that multiple regulatory mechanisms interact. Regulation of this gene is remarkable for both its rapidity and multiplicity of control mechanisms. The present and other recent studies indicate that Tf receptors are controlled at the level of gene transcription.³⁰ Recently, the 5' noncoding regulatory regions of the Tf receptor gene have been cloned and sequenced and appear to control serum-stimulated Tf receptor gene transcription.³¹ Specific binding of proteins to this region has been demonstrated,³¹ and in the future, it may be possible to determine whether the same or different proteins mediate Tf receptor gene expression in these diverse settings. In mouse cells transfected with mutant human Tf receptor constructs, the 3' noncoding regions also appear to regulate Tf receptor expression in response to iron.³² Further studies will determine how these regions of the Tf receptor gene interact during regulation of Tf receptor gene expression.

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Neutrophil Elastase Produces 52-kD and 30-kD Glucocorticoid Receptor Fragments in the Cytosol of Human Leukemia Cells

By Clark W. Distelhorst, Karen E. Janiga, Kathryn J. Howard, Sarah E. Strandjord, and Edward J. Campbell

Characterization of glucocorticoid receptors in leukemia cells is important to understand mechanisms of glucocorticoid resistance but has been impeded by receptor fragmentation in cytosol extracts. We recently found that formation of 52- and 30-kilodalton (kD) glucocorticoid receptor fragments in cytosol of leukemia cells is due to proteolysis and is blocked by diisopropylfluorophosphate (DFP). In the present study, we identify a 28-kD serine protease in cytosol of leukemia cells that binds [³H]DFP and correlates with the formation of 52- and 30-kD receptor fragments. This protease is immunoprecipitated by antiserum to neutrophil elastase. Limited digestion of [³H]dexamethasone-21-mesylate-labeled receptors by purified neutrophil elas-

tase produces 52- and 30-kD receptor fragments. Receptor fragmentation in the cytosol of leukemia cells is inhibited by methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethylketone, a highly specific inhibitor of neutrophil elastase. The addition of as few as 5% neutrophils to a lymphoid cell suspension provides sufficient elastase to produce receptor fragmentation. Our findings indicate that neutrophil elastase is responsible for receptor fragmentation in the cytosol of leukemia cells. The neutrophil elastase may be endogenous to the leukemia cells or may come from neutrophils that contaminate leukemia cell suspensions.

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THE EFFECTS OF STEROID HORMONES on cells are mediated through receptors that bind steroid hormones with both high affinity and a high degree of specificity.¹⁻³ Steroid-receptor complexes interact with specific DNA sequences in the genome and thereby regulate gene transcription.^{4,5} Steroid hormone receptors, including glucocorticoid receptors, have been characterized in a wide variety of cells and tissues and have been found to be notoriously subject to proteolysis.^{6,7} Although proteolysis of glucocorticoid receptors is generally considered to be responsible for receptor degradation, protease inhibitors have been shown to affect various aspects of receptor function, thus leading to postulates about the physiological role of proteolysis in receptor function.⁸ Limited proteolytic digestion of glucocorticoid receptors has been useful in defining the structure of the glucocorticoid receptor since exogenous proteases, specifically trypsin and chymotrypsin, separate the receptor into distinct functional domains and localize the steroid-binding and DNA-binding sites to relatively small segments of the receptor molecule.⁹⁻¹⁴

We recently found that cytosol prepared from fresh

human leukemia cells contains a serine protease that digests the normal-sized 97-kilodalton (kD) glucocorticoid receptor to produce 52- and 30-kD receptor fragments, each containing the steroid-binding site.¹⁵ We considered it to be of both fundamental and clinical importance to identify this protease. First, the protease is of fundamental interest because it digests the glucocorticoid receptor at a different site than chymotrypsin or trypsin.¹⁵ This protease may therefore be useful in further characterization of glucocorticoid receptor structure by limited proteolysis. Also, the major receptor fragment produced by this protease is very close in size to the nuclear transfer-increased mutant receptor that occurs in certain glucocorticoid-resistant mouse lymphoma cell lines.¹⁶ Therefore, this protease may be useful in investigating the nature of the mutant receptor. Second, the protease is of clinical interest because of the need to accurately quantify and characterize glucocorticoid receptors in the cytosol of human leukemia cells. Based on the work of other investigators, it is thought that proteolysis gives rise to artifactual abnormalities of glucocorticoid receptors in human leukemia cells.¹⁷⁻²⁰ This problem must be overcome to determine whether glucocorticoid receptor defects are a mechanism of glucocorticoid resistance in human leukemia cells.

Therefore, the present study was undertaken to identify the serine protease that is responsible for fragmentation of glucocorticoid receptors in the cytosol of human leukemia cells. The experimental approach taken in this study was based on our earlier observation that the potent serine protease inhibitor diisopropylfluorophosphate (DFP)¹ prevents proteolysis of glucocorticoid receptors in the cytosol of human leukemia cells.¹⁵ Since DFP binds irreversibly to the active site of serine proteases,^{21,22} we used [³H] DFP to label serine proteases in the cytosol of human leukemia cells and thereby determined the molecular weight (mol wt) of the serine protease that is responsible for receptor fragmentation. Based on this information, we performed a series of experiments that indicate that neutrophil elastase is the serine protease that produces 52- and 30-kD glucocorticoid receptor fragments in the cytosol of human leukemia cells. This finding has broad implications not only for investigation of glucocorticoid receptors in human leukemia cells but also for investigation of different types of steroid hormone receptors in a variety of cells and tissues.

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METHODS

Materials. Chemicals for gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA. The low-mol wt standards for gel electrophoresis were from Pharmacia Fine Chemicals, Piscataway, NJ. Neutrophil elastase and cathepsin G were purified to homogeneity from purulent sputum.²³ Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-chloromethylketone (MeOSucAlaAlaProValCH₂Cl) was purchased from Enzyme Systems Products, Livermore, CA, and was dissolved in methanol and stored at 4°C as a 6 mmol/L stock solution. Sheep antiserum to human neutrophil elastase and preimmune serum were purchased from ICN Immunobiologicals, Lisle, IL. Protein A-Sepharose, unlabeled dexamethasone, and other chemicals were from Sigma Chemical Co, St Louis. [6,7-³H] dexamethasone-21-mesylate (48.9 Ci/mmol) and [1,3-³H] diisopropylfluorophosphate, 3.4 Ci/mmol, ([³H]DFP) were from New England Nuclear, Boston.

Buffers. Phosphate-buffered saline, pH 7.8, contained 140 mmol/L NaCl, 3 mmol/L KCl, 10 mmol/L Na₂HPO₄, 15 mmol/L KH₂PO₄. The hypotonic buffer used to prepare cytosol contained 25 mmol/L Tris, pH 8.2, 1 mmol/L EDTA, 10% glycerol, and 20 mmol/L sodium molybdate. The sample buffer for gel electrophoresis contained 0.6 mol/L Tris, pH 8.85, 2% sodium dodecyl sulfate (SDS), 5% (vol/vol) β -mercaptoethanol, 10% (vol/vol) glycerol, and bromphenol blue. Buffer A, used in DNA-cellulose binding assays, contained 20 mmol/L Tris pH (7.8), 1 mmol/L EDTA, 50 mmol/L NaCl, 2 mmol/L β -mercaptoethanol, 20 mmol/L sodium molybdate, and 10% glycerol.

Cell lines. The IM-9 cell line is a B lymphoblast cell line that has a relatively high level of functional glucocorticoid receptors but is not killed by glucocorticoids.^{24,25} IM-9 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum at 37°C in a 5% CO₂/95% air atmosphere. Cells were used for experiments during the log phase of growth.

Leukemia cells. Samples of leukemia cells were isolated from peripheral blood or bone marrow by Ficoll-Hypaque separation as described in detail earlier.¹⁶ After separation, differential cell counts were performed on cells that were fixed to glass slides by cytocentrifugation and stained with Wright's stain. Leukemia cells were used for experiments immediately or after overnight incubation at 4°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum.

Neutrophils. Leukocytes were isolated from peripheral blood of normal healthy donors as described previously.²⁶ Whole blood was defibrinated by allowing the blood to clot around glass beads, thereby removing most of the platelets. The blood was then allowed to sediment in dextran to remove red cells, and the white cell-rich supernatant was sedimented through Ficoll-Hypaque gradients. Neutrophils were recovered from the bottom of the gradients just above the red cell pellet. Red cells were removed by hypotonic lysis in ammonium chloride.²⁷

Receptor labeling. The method of covalently labeling glucocorticoid receptors with [³H] dexamethasone-21-mesylate has been described in detail.¹⁶ Briefly, cells were washed and resuspended in phosphate-buffered saline. The cell suspensions were incubated in the presence or absence of 100-fold excess unlabeled dexamethasone with a concentration of [³H] dexamethasone-21-mesylate that is sufficient to saturate most receptor sites (see figure legends and Discussion). After incubation at 4°C for four hours, the cells were pelleted and the supernatant buffer discarded. Cytosol was prepared by freezing the cell pellets and then thawing the cell pellets in hypotonic buffer. After centrifuging the cytosol in an Eppendorf microfuge at 4°C for 15 minutes, it was mixed with sample buffer for gel electrophoresis and heated at 100°C for two minutes before

SDS-polyacrylamide gel electrophoresis (PAGE). Cytosol from 10 to 20 million cells was placed on each lane of gels.

Protease labeling. Cytosol was prepared by freeze-thaw lysis in hypotonic buffer as described earlier. [³H]DFP (3.4 Ci/mmol) was added to 20 μ L cytosol to give a final concentration of 200 μ Ci/mL. After the cytosol was incubated for 15 minutes at 4°C, it was heated at 100°C for two minutes and then subjected to SDS-PAGE.

Gel Electrophoresis. Cytosol was subjected to SDS-PAGE under reducing conditions as described previously in detail.^{16,28} Gels were stained with Coomassie blue dye and incubated with 1 mol/L sodium salicylate before autoradiography.²⁸ Mol wt standards were phosphorylase b (97.4 kD), bovine serum albumin (67 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α -lactalbumin (14.4 kD).²⁸

Limited proteolysis of receptors. Receptors in IM-9 cells were covalently labeled with [³H] dexamethasone-21-mesylate, and cytosol was prepared as described earlier. Neutrophil elastase was dissolved in phosphate-buffered saline at a concentration of 1 mg/mL and stored at 4°C. Neutrophil elastase was added to the cytosol at final concentrations that are described in the text, and then the cytosol was incubated for 15 minutes at 4°C. The reaction was stopped by adding the sample buffer for gel electrophoresis and heating the sample at 100°C for two minutes before SDS-PAGE.

Immunoprecipitation. Cytosol was prepared from leukemia samples or neutrophil samples in hypotonic buffer and labeled with [³H]DFP as described earlier. Aliquots (10 to 20 μ L) of cytosol prepared from 10 million cells were incubated with an equal volume of undiluted antielastase immune serum or preimmune serum at 4°C for 12 hours. An equal volume of protein A-Sepharose (50% vol/vol in hypotonic buffer) was then added and mixed by gentle rotation for two hours at 4°C. The protein A-Sepharose was then washed three times with hypotonic buffer and then three times with hypotonic buffer supplemented with 500 mmol/L NaCl and 0.2% Triton X-100. The protein A-Sepharose pellet was then heated at 100°C for five minutes in the sample buffer for SDS-PAGE. Insoluble precipitates were pelleted by centrifuging the samples at 200 g for ten minutes at 25°C. The clear supernatant was subjected to SDS-PAGE and analyzed by autoradiography as described earlier.

RESULTS

Detection of receptor fragments in leukemia cytosol. The mol wt of [³H] dexamethasone-21-mesylate-labeled glucocorticoid receptors in the cytosol of freshly isolated human leukemia cells was determined by SDS-PAGE. Representative experiments are shown in Figs 1 and 2. Acute lymphoblastic leukemia cells isolated from the bone marrow of two newly diagnosed patients were used in the experiment shown in Fig 1. The cell suspension isolated from the bone marrow of patient A (left panel, Fig 1) consisted of 93% lymphoblasts, 1% mature lymphocytes, 1% neutrophil precursors, 2% mature neutrophils, and 3% erythroid precursors. The cell suspension isolated from the bone marrow of patient B (right panel, Fig 1) consisted of 97% lymphoblasts, 1% mature lymphocytes, 1% mature neutrophils, and 1% erythroid precursors. Cell suspensions were incubated with [³H] dexamethasone-21-mesylate to label receptors in vivo, and then cytosol extracts were subjected to SDS-PAGE. Multiple proteins in cells bind [³H] dexamethasone-21-mesylate and therefore appear as bands on autoradiograms of gels. To distinguish the specific binding proteins or receptors from the nonspecific binding proteins, duplicate cell suspensions were incubated with [³H] dexamethasone-

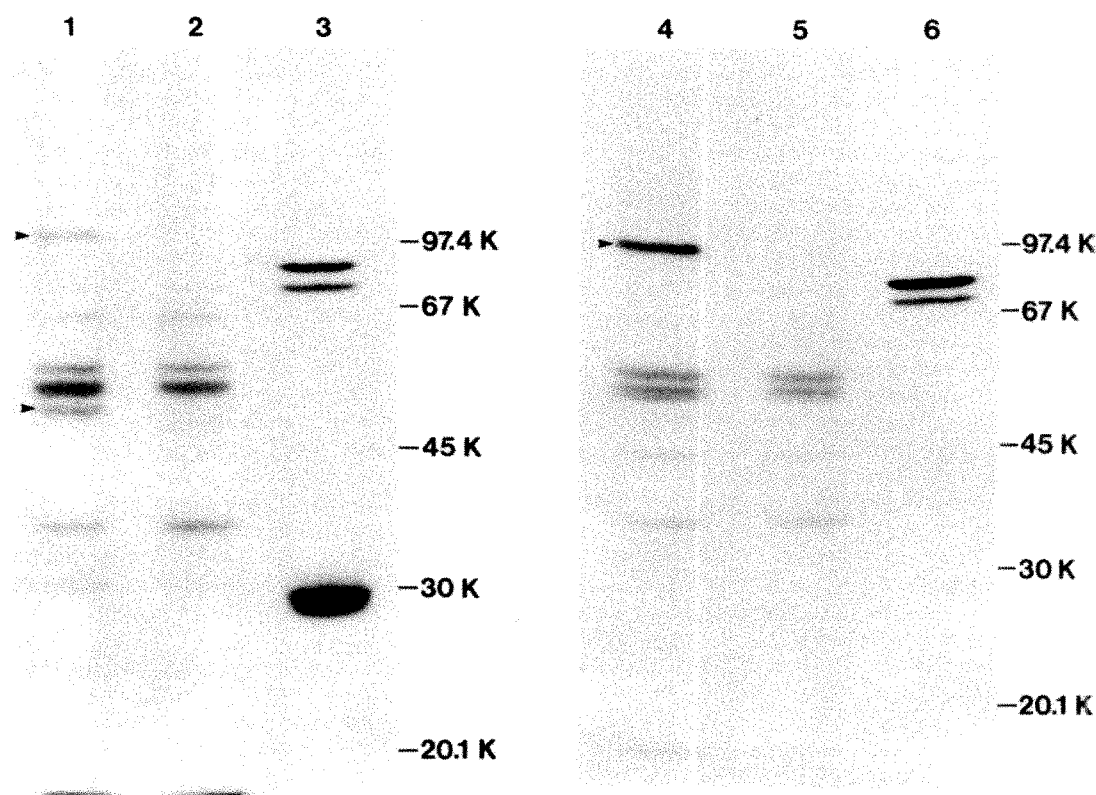


Fig 1. SDS-PAGE of cytosol proteins labeled with [^3H] dexamethasone-21-mesylate or [^3H]DFP. Lymphoblasts were isolated from the bone marrow of two patients with acute lymphoblastic leukemia, shown separately as left and right panels in this figure. Cells were incubated with 200 nmol/L [^3H] dexamethasone-21-mesylate to label glucocorticoid receptors, and then cytosol from the cells was subjected to SDS-PAGE (lanes 1, 2, 4, and 5). Unlabeled cytosol from duplicate sets of cells was incubated with [^3H]DFP to label serine proteases and then was subjected to SDS-PAGE (lanes 3 and 6). Cytosol from 28 million cells was applied to each lane in the left panel, and cytosol from 40 million cells was applied to each lane in the right panel. Autoradiograms shown in each panel were exposed for three days. The bands that correspond to receptors or receptor fragments are designated by arrowheads and can be distinguished from nonspecifically labeled proteins because they are not seen when labeling takes place in the presence of 100-fold excess unlabeled dexamethasone (lanes 2 and 5) but are seen when labeling takes place in the absence of competing unlabeled dexamethasone (lanes 1 and 4). Cytosol from one set of leukemia cells (left panel) contains both intact 97-kD glucocorticoid receptors and 50-kD receptor fragments, but cytosol samples from the other set of leukemia cells (right panel) contain only intact 97-kD receptors. A 28-kD protein that binds [^3H]DFP is detected in the cytosol from one set of leukemia cells (left panel, lane 3) but is barely visible in the cytosol from the other set of leukemia cells (right panel, lane 6). Mol wt standards are shown at the right of each panel where the designation 97.4 K means 97.4 kD.

21-mesylate in the presence or absence of 100-fold excess unlabeled dexamethasone. On autoradiograms, band(s) that correspond to labeled receptors or receptor fragments are seen only in the absence of competing unlabeled steroid (lane -) and are not seen in the presence of competing unlabeled steroid (lane +). Intact receptors having a mol wt of 97 kD were present in the cytosol of both leukemia samples (left and right panels, Fig 1). A 52-kD receptor fragment and a faint 30-kD receptor fragment are detected in the cytosol of one of the leukemia samples (left panel) but not in the cytosol of the other leukemia sample (right panel). The identity of the nonspecifically labeled proteins in the cytosol of the leukemia cells is unknown. As shown previously, the addition of DFP to the hypotonic buffer before cytosol preparation inhibited the formation of 52- and 30-kD receptor fragments in the cytosol of leukemia cells.¹⁵

Leukemia cell samples from 65 different patients have been studied, and 52-kD receptor fragments were detected in the cytosol of 27 of these samples.^{15,16} Glucocorticoid recep-

tor fragments that have a mol wt of 30 kD were detected in four of the 65 leukemia cell samples (one acute lymphoblastic leukemia, three acute nonlymphoblastic leukemias), as illustrated by the experiment shown in Fig 2. In this experiment, cells were isolated from the bone marrow of a patient with acute lymphoblastic leukemia in relapse. The cell suspension consisted of 64% lymphoblasts, 3% mature lymphocytes, 9% neutrophil precursors, 8% mature neutrophils, and 16% erythroid precursors. Only 30-kD receptor fragments and no intact receptors were detected in the cytosol prepared from this cell suspension (Fig 2).

Evidence that a 28-kD serine protease produces the receptor fragments. Since DFP binds irreversibly to the active site of serine proteases,^{21,22} cytosol from leukemia samples was incubated with [^3H]DFP and analyzed by SDS-PAGE. It should be noted that [^3H]DFP can bind to some proteins that are not proteases.^{21,29} Therefore, not all the [^3H]DFP-labeled proteins detected in the following experiments are necessarily serine proteases. Under the

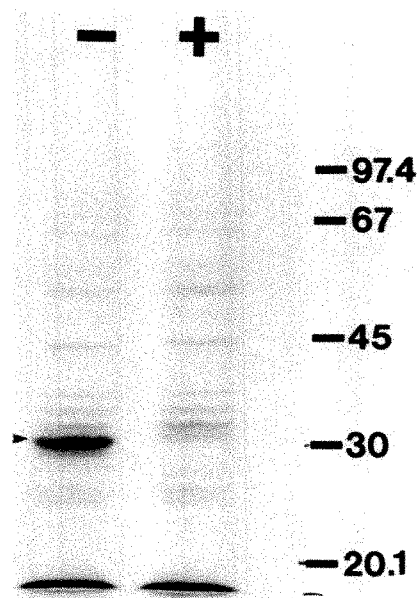


Fig 2. SDS-PAGE of cytosol proteins labeled with [^{3} H] dexamethasone-21-mesylate. Cells were isolated from the bone marrow of a patient with acute lymphoblastic leukemia. Duplicate cell suspensions were incubated with 200 nmol/L [^{3} H] dexamethasone-21-mesylate in the absence (lane -) or presence (lane +) of 100-fold excess unlabeled dexamethasone. Cytosol from the cells was then subjected to SDS-PAGE. Each lane on the gel represents the cytosol obtained from 32 million cells. An autoradiogram of the gel, exposed for three days, is shown here. An arrowhead points to a 30-kD receptor fragment. Mol wt standards are given at the right of the figure where the designation 97.4 means 97.4 kD.

labeling conditions used here, three major [^{3} H]DFP-labeled proteins having mol wts of 80, 75, and 28 kD were detected in cytosol samples (Fig 1). The mobility of these proteins was the same under reducing and nonreducing conditions (not shown). Based on the relative intensity of these proteins on autoradiograms, the amount of the 80- and 75-kD proteins in the two cytosol samples shown in Fig 1 appeared to be similar, but the amount of the 28-kD protein appeared to be much greater in the cytosol sample that contained 52-kD receptor fragments (left panel) than in the cytosol sample that did not contain 52-kD receptor fragments (right panel). The correlation between the relative amount of 28-kD [^{3} H]DFP-labeled protein and formation of 52-kD receptor fragments was confirmed in seven experiments using leukemia cells from a different patient for each experiment, thereby suggesting that the mol wt of the serine protease responsible for receptor digestion was 28 kD. The identity of the 80- and 75-kD [^{3} H]DFP-labeled proteins seen on autoradiograms is unknown.

One leukemia sample in which 30-kD receptor fragments were detected was labeled with [^{3} H]DFP and analyzed by SDS-PAGE (Fig 3). This sample was from a patient with acute nonlymphoblastic leukemia and consisted of 12% myeloblasts, 38% neutrophil precursors, 23% mature neutrophils, 18% mature lymphocytes, 3% monocytes, and 6% erythroid precursors. Only 30-kD receptor fragments were detected in the cytosol sample (Fig 3). A replicate sample of this cytosol appeared to contain a relatively large amount of

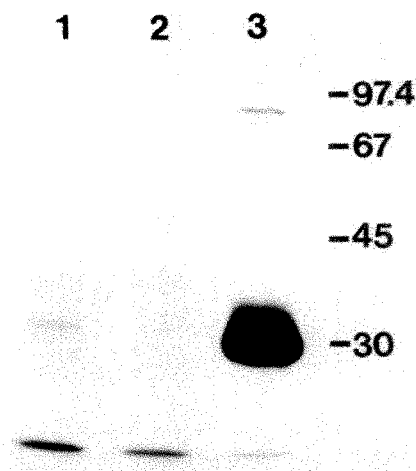


Fig 3. SDS-PAGE of cytosol proteins labeled with [^{3} H] dexamethasone-21-mesylate or [^{3} H]DFP. Myeloblasts were isolated from the bone marrow of a patient with acute nonlymphoblastic leukemia. Cells were incubated with 200 nmol/L [^{3} H] dexamethasone-21-mesylate in the absence (lane 1) or presence (lane 2) of 100-fold excess unlabeled dexamethasone to label glucocorticoid receptors, and then the cytosol was subjected to SDS-PAGE. Cytosol prepared from another aliquot of cells was incubated with [^{3} H]DFP to label serine proteases and then subjected to SDS-PAGE (lane 3). Each lane on the gel represents the cytosol obtained from 10 million cells. The autoradiogram was exposed for 14 days. A band that corresponds to a 30-kD receptor fragment is detected in the absence of competing unlabeled dexamethasone (lane 1) but not in the presence of competing unlabeled dexamethasone (lane 2). A [^{3} H]DFP-labeled protein that has a mol wt of ~30 kD is detected in the cytosol (lane 3). The autoradiogram of this [^{3} H]DFP-labeled protein was overexposed to visualize the receptor fragment in lane 1. This [^{3} H]DFP-labeled protein was more distinct on a shorter exposure of the same autoradiogram and corresponded to a mol wt of 28 kD (not shown). Mol wt standards are given at the right of the figure where the designation 97.4 means 97.4 kD.

a 28-kD [^{3} H]DFP-labeled protein, which suggests that a 28-kD serine protease may also be responsible for producing 30-kD receptor fragments.

Neutrophil elastase and cathepsin G, both serine proteases known to be present in normal leukocytes and leukemia cells, have a mol wt of 28 kD.³⁰ As shown in Fig 4, the 28-kD [^{3} H]DFP-labeled protein in the cytosol of leukemia cells was immunoprecipitated by antibodies to human neutrophil elastase. In this experiment, cells were isolated from the peripheral blood of a patient with acute nonlymphoblastic leukemia, and the cell suspension consisted of 74% myeloblasts, 7% neutrophils, and 19% mature lymphocytes.

Addition of neutrophils to human lymphoblasts produces 52- and 30-kD receptor fragments. Neutrophils are a rich source of elastase.^{31,32} In the mixing experiment shown in Fig 5, cultured human lymphoblasts (IM-9 cells) were incubated with [^{3} H] dexamethasone-21-mesylate to label the glucocorticoid receptors. Unlabeled neutrophils were then added to the labeled IM-9 cells immediately before cytosol preparation and SDS-PAGE. It should be noted that the neutrophils were preincubated with 20 μ mol/L unlabeled dexamethasone for four hours at 4°C to prevent their receptors from being labeled with [^{3} H] dexamethasone-21-mesylate.

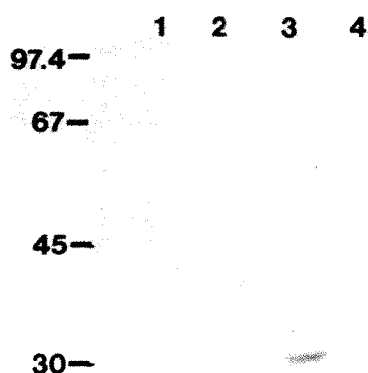


Fig 4. Immunoprecipitation of the [^3H]DFP-labeled protein by antielastase antiserum. Cytosol was prepared from 14 million neutrophils in 20 μL of hypotonic buffer, and cytosol was prepared from 10 million acute nonlymphoblastic leukemia cells in 20 μL of hypotonic buffer. The cytosol samples were incubated with [^3H]DFP for 15 minutes at 4°C and then divided into two equal parts for immunoprecipitation with immune serum or preimmune serum as described in Methods. Immunoprecipitates were analyzed by SDS-PAGE, and shown here is an autoradiogram of the gel exposed for four days. Lane 1, neutrophil cytosol, antielastase antiserum; lane 2, neutrophil cytosol, preimmune serum; lane 3, leukemia cytosol, antielastase antiserum; lane 4, leukemia cytosol, preimmune serum. Mol wt standards are given at the left of the figure where the designation 97.4 means 97.4 kD.

Cytosol from replicate unlabeled cell mixtures were incubated with [^3H]DFP for 15 minutes at 4°C before SDS-PAGE. As shown in Fig 5, the presence of as few as 5% neutrophils in the cell mixture produced a detectable 28-kD [^3H]DFP-labeled protein and a 52-kD receptor fragment. The presence of larger proportions of neutrophils in cell mixtures produces both 52- and 30-kD receptor fragments (Fig 5).

Digestion of receptors by purified neutrophil elastase produces 52- and 30-kD receptor fragments. [^3H] dexamethasone-21-mesylate-labeled glucocorticoid receptors in cytosol from IM-9 cells were subjected to limited digestion by purified neutrophil elastase (Fig 6). Limited digestion of receptors by human leukocyte elastase at a concentration of 10 $\mu\text{g}/\text{mL}$ produced 52-kD receptor fragments. Both 52- and 30-kD receptor fragments were produced at an elastase concentration of 20 $\mu\text{g}/\text{mL}$, and only 30-kD receptor fragments were detected at an elastase concentration of 50 $\mu\text{g}/\text{mL}$. This pattern of receptor digestion by elastase was confirmed in multiple experiments. Receptor digestion by purified neutrophil elastase was blocked by preincubating the elastase used in these experiments with MeOSucAlaAla-ProValCH₂Cl, a potent and specific inhibitor of neutrophil elastase³³ (Fig 7). We showed previously that limited digestion of [^3H] dexamethasone-21-mesylate-labeled receptors in the cytosol of fresh human leukemia cells by chymotrypsin produces a 40-kD receptor fragment.¹⁵ Limited digestion of [^3H] dexamethasone-21-mesylate-labeled receptors in IM-9 cytosol by cathepsin G also produced 40-kD receptor fragments (unpublished data).

A specific elastase inhibitor blocks receptor digestion in leukemia cytosol. In the experiment shown in Fig 8, unlabeled cytosol from a leukemia sample (the same sample used

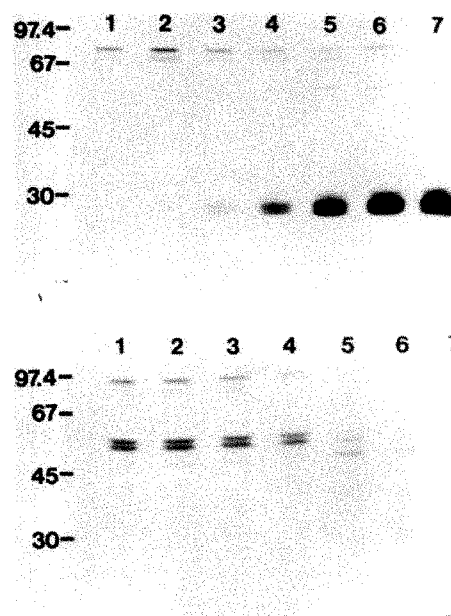


Fig 5. Mixing studies. (Top panel) IM-9 cells were mixed with neutrophils and then cytosol was prepared. The cytosol was incubated with [^3H]DFP for 15 minutes at 4°C to label serine proteases and was then subjected to SDS-PAGE. An autoradiogram of the gel, exposed for four days, is shown. (Bottom panel) IM-9 cells were incubated with 25 nmol/L [^3H] dexamethasone-21-mesylate to label glucocorticoid receptors. At the end of the incubation period, unlabeled neutrophils were added to the IM-9 cells, and then the cytosol was immediately prepared and subjected to SDS-PAGE. An autoradiogram of the gel is shown. Another aliquot of cells was incubated with [^3H] dexamethasone-21-mesylate in the presence of 100-fold excess unlabeled dexamethasone to distinguish between specifically labeled (receptor) proteins and nonspecifically labeled proteins (not shown). The percentage of neutrophils in each sample was as follows: lane 1, 0%; lane 2, 2%; lane 3, 5%; lane 4, 10%; lane 5, 20%; lane 6, 30%; lane 7, 40%. [^3H]DFP-labeled proteins that have a mol wt of ~28 kD are detected when the cell sample contains 5% neutrophils (lane 3, top panel) and become progressively more intense with increasing concentrations of neutrophils. Fifty-kilodalton receptor fragments are detected when cell samples contain 5% neutrophils (lane 3, bottom panel) and reach maximum intensity when cell samples contain 20% neutrophils (lane 5, bottom panel). Normal-sized 97-kD receptors are not detected when cell samples contain 25% or greater neutrophils. Thirty-kilodalton receptor fragments are detected when cell samples contain high proportions of neutrophils (lanes 5, 6, and 7, bottom panel). Mol wt standards are shown along the left side of the figure where the designation 97.4 means 97.4 kD.

in the experiments shown in Fig 4) was gently mixed with [^3H] dexamethasone-21-mesylate-labeled cytosol from IM-9 cells and then incubated for one hour at 4°C before SDS-PAGE, which resulted in the formation of 52-kD receptor fragments. It should be noted that the unlabeled leukemia cells were preincubated with 20 $\mu\text{mol}/\text{L}$ unlabeled dexamethasone for four hours at 4°C to prevent their receptors from being labeled with [^3H] dexamethasone-21-mesylate. Preincubation of the unlabeled cytosol with 500 $\mu\text{mol}/\text{L}$ MeOSucAlaAlaProValCH₂Cl for 30 minutes at 4°C before addition to the labeled IM-9 cytosol prevented the formation of 52-kD receptor fragments (Fig 8). The addition of 500 $\mu\text{mol}/\text{L}$ MeOSucAlaAlaProValCH₂Cl to the unlabeled cytosol immediately before or shortly after addition to the

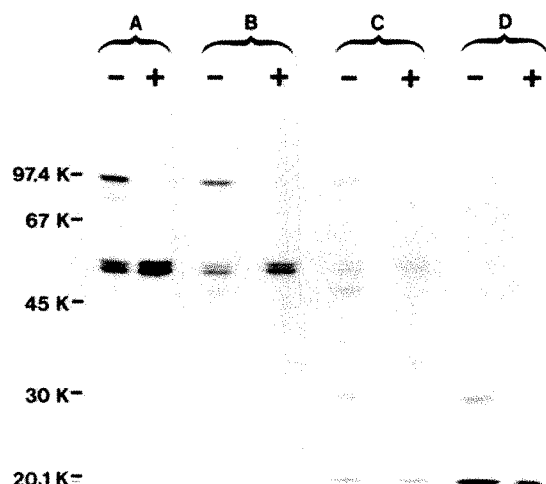


Fig 6. Receptor digestion by purified neutrophil elastase. IM-9 cells were incubated with 25 nmol/L [^{3} H] dexamethasone-21-mesylate in the absence (– lanes) or presence (+ lanes) of 100-fold excess unlabeled dexamethasone to label glucocorticoid receptors, and then cytosol was prepared as described in Methods. Twenty-microliter cytosol samples were incubated at 4°C for 15 minutes without elastase (A) or with elastase at concentrations of 10 (B), 20 (C) or 50 μ g/mL (D). The cytosol samples were then immediately subjected to SDS-PAGE. Each lane contains the amount of cytosol derived from 10 million cells. An autoradiogram of the gel (exposed for six days) is shown here. The radiolabeled bands that represent intact receptors or receptor fragments are seen only in the absence of competing unlabeled dexamethasone (lane –) and not in the presence of competing unlabeled dexamethasone (lane +), whereas nonspecifically labeled proteins are detected both in the presence and absence of unlabeled dexamethasone. Intact 97-kD receptors are seen in the absence of elastase (A) and with progressively diminishing intensity after incubation with elastase (B, C, and D). Fifty-kilodalton receptor fragments (B and C) and 30-kD receptor fragments (C and D) are detected after incubation with elastase. Two faint bands that appear to represent specifically labeled proteins having mol wts of 79 and 43 kD are detected in lanes marked A and B. These bands presumably represent receptor fragments and were not detected in all experiments. Mol wt standards are shown at the right of the figure where the designation 97.4 K means 97.4 kD.

labeled IM-9 cytosol did not prevent receptor degradation (not shown).

DISCUSSION

Glucocorticosteroids are among the most effective agents for the treatment of lymphoid leukemias and lymphomas. However, malignant lymphoid cells are often resistant to the cytotoxic effects of glucocorticoids at the time of diagnosis or become resistant to glucocorticoids during the course of therapy.^{34,35} Mechanisms of glucocorticoid resistance have been studied extensively in mouse lymphoma cell lines, and several different glucocorticoid receptor defects that give rise to glucocorticoid resistance have been defined.² Mechanisms of glucocorticoid resistance in human leukemia and lymphoma cells are less well understood. Although the levels of glucocorticoid receptors in human leukemia and lymphoma cells have been shown to correlate with response to treatment and overall survival, a deficiency of glucocorticoid receptors is an uncommon cause of glucocorticoid resistance in human leukemia.³⁶⁻⁴¹ When leukemia cells from glucocorticoid-resis-

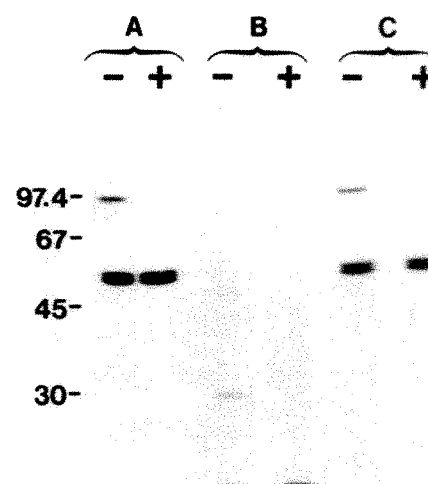


Fig 7. Effect of elastase inhibitor on receptor digestion by purified neutrophil elastase. [^{3} H] dexamethasone-21-mesylate-labeled cytosol was prepared from IM-9 cells and digested with elastase as described in Fig 6. (A) No elastase, (B) 50 μ g/mL elastase, (C) 50 μ g/mL elastase that was preincubated for 30 minutes at 4°C with MeOSucAlaAlaProValCH₂Cl before addition to labeled IM-9 cytosol. Bands on the autoradiogram that correspond to receptors or receptor fragments are detected in the absence (– lanes) but not in the presence (+ lanes) of competing unlabeled dexamethasone. Receptor digestion with 50 μ g/mL elastase produced 50- and 30-kD receptor fragments (B). Only intact 97-kD receptors are detected in control cytosol (A) or in cytosol that was incubated with elastase that had been preincubated with MeOSucAlaAlaProValCH₂Cl. Mol wt standards are shown to the right of the figure where the designation 97.4 means 97.4 kD.

tant patients have been studied, they generally have been found to contain adequate numbers of glucocorticoid receptors, which suggests that glucocorticoid resistance is due to functionally defective receptors or is due to a defect at a subsequent step in the response of cells to glucocorticoids. Although functionally defective glucocorticoid receptors have been described in human leukemia cells, it is now thought that these abnormalities are likely to be artifacts secondary to glucocorticoid receptor degradation by endogenous proteases.¹⁷⁻²⁰ Therefore, it is important to identify the protease responsible for receptor fragmentation and to develop a means of stabilizing the receptors so that the properties of glucocorticoid receptors in leukemia cells can be accurately determined.

Proteolysis of glucocorticoid receptors in the cytosol of human leukemia cells isolated from peripheral blood or bone marrow has been studied extensively. Stevens et al⁴² showed that glucocorticoid receptors in chronic lymphocytic leukemia cells are often degraded. Sherman et al¹⁷ detected both intermediate and small glucocorticoid receptor fragments in lymphoma cells and a variety of types of leukemia cells. The latter investigators also found similar glucocorticoid receptor fragments in rat liver and kidney cytosol, thereby indicating that proteases responsible for receptor degradation are not unique to leukemia or lymphoma cells.⁶ They have isolated a protease from rat kidney that is capable of cleaving glucocorticoid receptors from human leukemia cells.⁷ Holbrook et al^{18,19} also detected the formation of glucocorticoid receptor fragments in the cytosol of human leukemia cells and found



Fig 8. Effect of elastase inhibitor on receptor proteolysis in leukemia cytosol. IM-9 cells were incubated with 25 nmol/L [^{3} H] dexamethasone-21-mesylate in the absence (– lanes) or presence (+ lanes) of 100-fold excess unlabeled dexamethasone, and then cytosol was prepared as described in Methods. Unlabeled cytosol from the same leukemia sample described in Fig 4 (equivalent of 10 million cells/20 μ L hypotonic buffer) was diluted either 1:5 or 1:2 with hypotonic buffer and then incubated for 30 minutes at 4°C with 500 μ mol/L MeOSucAlaAlaProValCH₂Cl or an equivalent volume of hypotonic buffer. Twenty-microliter aliquots of the unlabeled and diluted leukemia cytosol were then mixed with 20- μ L aliquots of [^{3} H] dexamethasone-21-mesylate-labeled cytosol from IM-9 cells (the equivalent of 10 million cells/20 μ L hypotonic buffer). The mixtures were incubated for one hour at 4°C and then subjected to SDS-PAGE. Autoradiograms of gels, exposed for four days, are shown here. (Top panel) Unlabeled cytosol was not preincubated with MeOSucAlaAlaProValCH₂Cl. (Bottom panel) Unlabeled cytosol was preincubated with MeOSucAlaAlaProValCH₂Cl. (A) Control in which no unlabeled cytosol was added to labeled IM-9 cytosol, (B) 1:5 dilution of unlabeled leukemia cytosol added to labeled IM-9 cytosol, (C) 1:2 dilution of unlabeled leukemia cytosol added to labeled IM-9 cytosol. Mol wt standards are shown to the left of the figure where the designation 97.4 means 97.4 kD. Arrowheads point to the intact receptor and receptor fragments.

that an endogenous factor in chronic lymphocytic leukemia cells is capable of stabilizing glucocorticoid receptors in the cytosol of leukemia cells. The same investigators subsequently found that this endogenous factor has the properties of calpastatin, a naturally occurring inhibitor of calpains (a family of neutral calcium-activated proteases), thus implicating calpains in the degradation of glucocorticoid receptors in the cytosol of human leukemia cells and other cells including rat thymocytes.⁴³

In the foregoing studies, the size of glucocorticoid receptors and glucocorticoid receptor fragments was determined under nondenaturing conditions by gel filtration.^{6,17} Recently, [^{3} H] dexamethasone-21-mesylate was developed as an irreversible affinity ligand of the glucocorticoid receptor, thus enabling the mol wt of glucocorticoid receptors to be determined under denaturing conditions by polyacrylamide gel electrophoresis (SDS-PAGE).^{44,45} This method not only

provides a more accurate determination of mol wt but is a more sensitive technique than gel filtration for detecting glucocorticoid receptor fragments.⁴⁶ We have now used this technique to characterize glucocorticoid receptors in the cytosol of leukemia cells from 65 different patients.^{15,16} In these studies, we affinity labeled the glucocorticoid receptors within intact cells with [^{3} H] dexamethasone-21-mesylate and then subjected cytosol from the cells to SDS-PAGE to determine the mol wt of intact receptors and receptor fragments that contain the steroid-binding site.^{15,16} The results of these studies demonstrated that 52- and 30-kD receptor fragments were formed during or after cytosol preparation due to the action of a serine protease.¹⁵ The size of these receptor fragments is similar to the receptor fragments described in earlier studies discussed in which the size of receptor fragments was measured by gel filtration.^{17,42} The mol wt of glucocorticoid receptors in all 65 samples was normal if DFP was included in the hypotonic buffer used for cytosol preparation, thus indicating that proteases that are not inhibited by DFP are unlikely to be responsible for receptor proteolysis under the assay conditions used.¹⁵

The present study indicates that neutrophil elastase is the serine protease that is responsible for the formation of 52- and 30-kD glucocorticoid receptor fragments in the cytosol of human leukemia cells. Although a number of different serine proteases are known to be present in mammalian cells, labeling studies using [^{3} H]DFP suggest that the protease responsible for glucocorticoid receptor fragmentation is a 28-kD protein (Figs 1 and 3). Both of the major leukocyte serine proteases, neutrophil elastase and cathepsin G, are 28-kD proteins.²³ The ability of antiserum to neutrophil elastase to immunoprecipitate a 28-kD [^{3} H]DFP-labeled protein from the cytosol of human leukemia cells suggests that the responsible protease might be neutrophil elastase (Fig 4). Two different types of experimental evidence indicate that neutrophil elastase produces 52- and 30-kD receptor fragments in the cytosol of human leukemia cells. First, limited digestion of [^{3} H] dexamethasone-21-mesylate-labeled receptors by purified neutrophil elastase produced 52- and 30-kD receptor fragments, an effect that was blocked by preincubating the elastase preparation with a potent and specific elastase inhibitor, MeOSucAlaAlaProValCH₂Cl²⁶ (Figs 6 and 7). Second, the receptor-degrading activity of leukemia cytosol was blocked by preincubating the cytosol with MeOSucAlaAlaProValCH₂Cl before addition to [^{3} H] dexamethasone-21-mesylate-labeled receptors (Fig 8).

The observation that neutrophil elastase digests the human glucocorticoid receptor to produce 52-kD fragments and that cathepsin G and chymotrypsin digest the receptor to produce 40-kD fragments is consistent with what is known about the primary structure of the human glucocorticoid receptor. The complementary DNA (cDNA) for the human glucocorticoid receptor has recently been cloned, and the complete amino acid sequence of the receptor has been deduced from the receptor cDNA.⁴⁷ Based on active site mapping of serine proteases by Harper et al,⁴⁸ the specific bonds in the glucocorticoid receptor that are cleaved by elastase and cathepsin G can be predicted. Elastase prefers substrates with relatively small alkyl side chains and prefer-

entially cleaves proteins at valyl bonds.⁴⁶ The 52-kD receptor fragment produced by elastase digestion of the glucocorticoid receptor binds [³H] dexamethasone-21-mesylate and therefore contains the steroid-binding domain of the receptor, which has been localized to the carboxy terminal half of the receptor molecule.⁴⁷ A valyl bond that is located in a peptide sequence optimal for elastase digestion is located at amino acid 359.⁴⁷ Digestion of the receptor at this position would produce a steroid-binding receptor fragment that has a calculated mol wt of 54 kD. Both chymotrypsin and cathepsin G prefer to cleave peptide sequences at bulky or aromatic amino acids.⁴⁸ Phenylalanines are located at positions 444 and 445 in the receptor sequence.⁴⁷ Digestion of the receptor at this position by either chymotrypsin or cathepsin G would produce a steroid-binding receptor fragment that has a calculated mol wt of 44 kD. Therefore, the size of receptor fragments determined by SDS-PAGE is predicted from the primary structure of the receptor molecule.

Based on the aforementioned information, it can be predicted that the 52-kD receptor fragment produced by elastase contains a ~10-kD region of the receptor molecule that is located near the DNA-binding domain of the receptor and that is not present in the chymotryptic fragment of the receptor. Limited digestion of receptors with neutrophil elastase may therefore be a useful tool for defining the function of this region of the receptor molecule. Since the 52-kD receptor fragment produced by neutrophil elastase is similar in size to the nuclear transfer-increased mutant glucocorticoid receptor in mouse lymphoma cells, limited digestion of wild-type mouse lymphoma cell glucocorticoid receptors with neutrophil elastase may also be useful for investigating the nature of the mutant receptor.

Mature neutrophils and neutrophil precursors contain relatively large amounts of elastase.^{31,32} Mixing studies in which neutrophils were added to [³H] dexamethasone-21-mesylate-labeled lymphoblasts (IM-9 cells) demonstrate that as few as 5% neutrophils can provide sufficient elastase

to generate 52-kD receptor fragments (Fig 5). This observation raises the question of whether the elastase that produces receptor fragmentation in the cytosol of leukemia samples comes from the leukemia cells themselves or from variable numbers of neutrophils that contaminate most leukemia cell samples. Neutrophil elastase has been detected by immunohistochemical assays in nonlymphoid leukemia cells but not in lymphoid leukemia cells.^{49,50} Therefore, it would seem likely that receptor fragmentation in lymphoid leukemia cells is due to elastase that arises from the small number of neutrophils that contaminate leukemia samples or that lymphoid leukemia cells contain an elastaselike protease that might not be detected by immunohistochemical assays that use antibodies directed toward neutrophil elastase. It is of interest in this regard that an elastolytic activity has been demonstrated to be associated with the cell membranes of mature human lymphocytes⁵¹ and that an enzyme identical to the elastase of neutrophils has been found in human monocytes and the monocytic leukemia cell line U937.⁵²

The observation that as few as 5% neutrophils in a cell suspension provides enough elastase to produce glucocorticoid receptor fragmentation has important implications for the study of glucocorticoid receptors in cytosol prepared from any tissue (eg, liver and kidney) where blood cell contamination is likely to occur. Future studies are planned to determine whether neutrophil elastase contributes to degradation of other types of steroid hormone receptors in human tissues. By inhibiting neutrophil elastase with either DFP or MeOSucAlaAlaProValCH₂Cl, it should now be possible to accurately determine the functional properties of glucocorticoid receptors in the cytosol of human leukemia cells in search of receptor defects that might give rise to glucocorticoid resistance.

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CONCISE REPORT

High-Dose Chemoradiotherapy and Autologous Bone Marrow Transplantation for Resistant Multiple Myeloma

By Bart Barlogie, Raymond Alexanian, Karel A. Dicke, Gunar Zagars, Gary Spitzer, Sundar Jagannath, and Leonard Horwitz

Seven patients with advanced multiple myeloma, refractory to therapy with alkylating agent-VAD (vincristine-adriamycin-dexamethasone), received a regimen combining high-dose melphalan with total body irradiation supported by autologous bone marrow transplantation. Very rapid, usually >90% tumor mass reduction was achieved in six patients, regardless of prior chemotherapy responsiveness and marrow plasmacytosis up to 30%. Despite signs of early relapse in three patients (median

remission duration of all patients, 15 months), five remain alive and well without further cytotoxic therapy from 2 to 21 months (median, 9+ months). Two patients died, one from surgical complications after transplantation and a second due to persistent neutropenia with fatal pneumonia. This treatment provides meaningful disease control for selected patients with resistant myeloma and a poor prognosis.

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FOR NEARLY 20 years, the combination of melphalan and prednisone (MP) has been the standard therapy for multiple myeloma. Once resistance to treatment develops, other agents have been ineffective, and survival has been short. Using a program that combines a continuous infusion of vincristine and adriamycin with dexamethasone (VAD), about 50% of patients resistant to MP responded, but for a median duration of only 9 months.^{1,2} Despite encouraging results with high-dose melphalan (HDM) in untreated myeloma,^{3,4} the median duration of remission in patients resistant to VAD was only 3 months.⁵ Since autologous bone marrow transplantation (BMT) improved the tolerance to HDM,⁵ we added total body irradiation (TBI) to the cytoreductive program^{5a} in an attempt to achieve more frequent and durable remissions in a tumor generally acknowledged to be radiosensitive. While there is only limited experience with TBI alone in myeloma,⁶ extramedullary plasmacytomas are dependably eradicated with total doses of 4500 cGy,⁷ and local manifestations of advanced myeloma are effectively palliated even with single doses of 700 to 1000 cGy.^{8,9}

We now report our initial experience with TBI and HDM in conjunction with autologous BMT in seven consecutive patients with VAD-refractory myeloma. Six patients have responded with >75% tumor mass reduction for a median of 15 months; and five patients remain alive from 2 to 21 months. Incidence and duration of remission were independent of prior chemotherapy resistance and not influenced by the degree of plasmacytosis in the marrow autografts.

MATERIALS AND METHODS

Seven consecutive patients with advanced multiple myeloma resistant to prior standard treatments and VAD form the basis of this report (Table 1). Their ages ranged from 45 to 63 years (median, 50 years). Written informed consent was obtained from all patients prior to therapy, indicating its potential benefits and risks, in keeping with institutional policy. Three patients were unresponsive to initial alkylating agent and VAD therapy, and one patient (ES) had not even responded to HDM at a dose of 70 mg/M²; four were relapsing despite VAD. Tumor mass was high in four patients and intermediate in two; myeloma protein type was IgA in three and IgG in three; one patient had nonsecretory myeloma with 27% plasmacytosis (patient JH). Four patients were moderately disabled from painful lumbar spine compression fractures, and one patient was bedridden from fractures of femur and humerus, with hypercalcemia and progressive skull plasmacytomas (Table 1, patient BL).

Except for the one bedridden patient who required intensive nursing, treatment was given in a protected environment unit and consisted of TBI in a dose of 850 cGy in five fractions over 2½ days, preceded (two patients, FS and GR, Table 1), or followed one day later by melphalan 140 mg/M² intravenously (IV) over 30 minutes (remaining five patients). Bone marrow was infused on the following day in a dose of at least 2×10^8 nucleated cells per kilogram body weight. Autologous marrow had been harvested during a previous VAD-induced remission in three patients (marrow plasmacytosis of 6%, 6%, and 11%) and after resistance to initial or salvage alkylating agent-VAD treatment in four patients (6%, 10%, 27%, and 30% plasmacytosis). Antibiotic prophylaxis was performed with trimethoprim sulfamethoxazole and ketoconazole. Additional supportive care was given as needed, including blood products and IV broad spectrum antibiotics. Frequent measurements of myeloma protein level were conducted, and clinical response was defined by a greater than 75% reduction in calculated tumor mass.¹⁰ Bone marrow aspirates and biopsies were examined morphologically and by flow cytometry to determine abnormalities in DNA and RNA content and to monitor the proportion of cells with monoclonal cytoplasmic immunoglobulin light chain expression.^{11,12} This technique has consistently detected <1% monoclonal plasma cells.

RESULTS

All six patients with measurable myeloma protein responded rapidly to treatment with a median tumor halving time of 12 days, and ≥90% tumor mass reduction was achieved in four patients (Table 1, Fig 1). Marrow plasmacytosis cleared in all seven patients, including two whose

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Table 1. Patient Characteristics and Response to TBI/High Dose Melphalan

Patient	Age (yr)	Months from Diagnosis	No. of Prior Responses	Tumor* Mass	Performance† Status	Tumor Cells in Marrow		Response		Current Status					
						Autograft (%)‡	Tumor Reduction (%)	Tumor Halving (d)	Relapse- Free (mo)	Survival (mo)	Tumor Cells in Bone Marrow (%)‡	Hemoglobin (g/dL)	Granulocytes (× 10 ³ /μL)	Platelets (× 10 ³ /μL)	
FS	46	4	0	I	2	30	93	12	21+	21+	<1	13.1	2.5	295	Yes
GR	50	106	3	H	2	6	99	9	15	17+	<1	14.3	2.9	171	Yes
LQ	51	6	0	I	0	10	78	12	11+	11+	<1	14.4	3.1	321	Yes
RS	45	27	1	H	0	6	90	12	6	8+	3	12.3	4.0	300	Yes
BL	59	31	1	H	4	11	97	10	3	3	<1	11.0	2.5	48	Dead, 3.0 mo
JH	50	39	1	H	0	27	<1% PC§	—	—	2.5	<1	11.7	0.7	10	Dead, 2.5 mo
ES	63	27	0	I	0	6	90	8	2+	2+	<1	10.3	3.5	165	Yes

*Tumor mass: I, intermediate; H, high (Durie-Salmon).

†Performance status, Zubrod scale.

‡Using DNA-Cig flow cytometry.

§Plasma cells, nonsecretory myeloma.

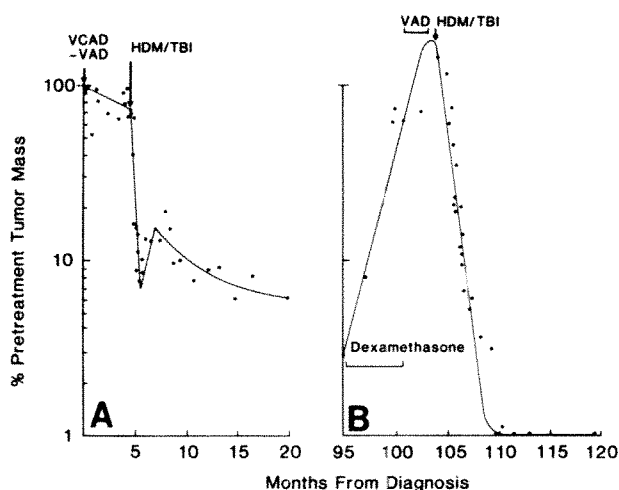


Fig 1. Tumor mass reduction in refractory myeloma following high-dose melphalan (HDM)/TBI and autologous bone marrow support. (A) Primary refractory disease. A 46-year-old patient failed to respond to initial VAD plus cyclophosphamide but responded to HDM/TBI despite marked plasmacytosis of 30% in the marrow autograft. (B) Third relapse. This 50-year-old patient had responded previously but achieved even greater cytoreduction after HDM/TBI.

autografts contained 27% and 30% plasma cells. One of these patients (JH) died from pneumonia 2½ months after treatment with poor marrow engraftment. A second patient (BL) with >90% cytoreduction died after 3 months (with early signs of relapse) from uncontrolled osteomyelitis, which had developed following surgical reduction of a femoral fracture. Three patients remain in remission without further therapy at 2, 11, and 21 months after chemoradiotherapy; the other two patients have developed signs of early relapse at 6 and 15 months, but currently do not require cytotoxic treatment. Five patients were discharged from the hospital within 2 months and have resumed normal activities.

Despite extensive prior therapy (including HDM alone in a 63-year-old patient, ES) and varying degree of marrow plasmacytosis, the hematologic recovery was prompt in six of seven patients treated. The median recovery time of granulocytes to 500/ μ L was 25 days (range, 19 to 62 days) and of platelets to 50,000/ μ L was 41 days (range, 20 to 99+ days). Within 3 months after treatment, granulocytes exceeded 1500/ μ L and platelets 100,000/ μ L in five of the seven patients; these five patients eventually achieved normal

hemograms with average values for hemoglobin of 12.9g%, for granulocytes of 3,200/ μ L and for platelets of 250,000/ μ L. In addition, their marrow biopsies and aspirates showed normal cellularity and differential counts, respectively, without detectable monoclonal plasmacytosis on flow cytometry. One patient (BL) remained thrombocytopenic at a level of 50,000/ μ L until his death, 3 months after therapy, from osteomyelitis and sepsis after surgical reduction of femoral fracture. A second patient (JH) with 27% plasma cells in the autograft had only partial engraftment with persistent neutropenia of <750/ μ L and severe thrombocytopenia <10,000/ μ L.

Four patients had fever without documented infection during the seven to ten days of agranulocytosis, prompting empiric administration of intravenous antibiotics. Three patients required ventilator support for pneumonia, two short-term (BL and ES), whereas the third patient (JH) with slow marrow engraftment died from interstitial pneumonitis, with marked pulmonary fibrosis and no evidence of myeloma at autopsy.

DISCUSSION

While the prognosis of individual patients is often uncertain, none of our patients was expected to live more than 8 months without disease control. Only 15 of 37 comparable patients had responded previously to HDM alone at doses ranging from 50 to 140 mg/M² (90 and 100 mg/M², 17 patients) with a relapse-free and overall survival of only 3 and 5 months, respectively (Table 2).⁵ In contrast, and stressing the cytoreductive potency of added TBI in plasma cell myeloma,⁶ HDM/TBI provided more frequent and marked cytoreduction with a median remission time of 15 months and five of seven patients surviving from 2 to 21 months (median, 9+ months). When administered without BMT, HDM was associated with a high early mortality rate of about 25%, which was reduced markedly by BMT support despite higher doses of cytotoxic therapy (Table 2). The poor marrow engraftment in one of our patients may be attributable to a high degree of plasmacytosis (27%), although a similar patient had prompt hematologic recovery and remains in remission with normal marrow and peripheral hemogram more than 1½ years after therapy.

Because of the reduced tolerance to graft-v-host disease with allogeneic BMT in older patients, marrow-ablative therapy has not been explored systematically in multiple myeloma. Cyclophosphamide/TBI and modifications with

Table 2. High-Dose Melphalan for Refractory Myeloma

HDM (mg/M ²)	TBI (850cGy)	BMT	N	Median Age (yr)	R	(CR)	ED	Median Survival (mo)
<90	—	—	13	62	3	(0)	4	4
90-100	—	—	17	46	8	(1)	4	4
140	—	Auto	7	63	4	(0)	1	6
140	+	Auto	7	50	6	(0)	1	Not reached
140	+	Allo	2	40	2	(2)	0	6, 14*

Abbreviations: HDM, high-dose melphalan; TBI, total body irradiation; BMT, bone marrow transplantation; N, no. of patients; R, patients with >75% tumor reduction including three achieving complete disappearance of M protein by immunofixation (CR); ED, early death; Auto, autologous; Allo, allogeneic bone marrow transplantation.

*Individual survival of two patients.

syngeneic or allogeneic BMT have been effective in about a dozen patients, many of whom still had drug-responsive myeloma.¹³⁻¹⁹ However, myeloma protein disappearance did not occur in any patient with progressive or unresponsive disease and was also not observed in the current study with TBI/HDM when supported by autologous BMT. The induction of complete remission in two additional patients with advanced and VAD-refractory myeloma receiving allogeneic BMT with TBI/HDM may suggest superior activity of HDM to high dose cyclophosphamide, as was evident from previous trials where only two of 15 patients responded briefly to high dose cyclophosphamide alone in contrast to a 48% response rate with HDM.^{4,20} Thus, HDM appears to be at least as effective as high dose cyclophosphamide when combined with TBI. One allogeneic BMT recipient died 6 months after therapy from pneumocystis carinii pneumonia with signs of early disease recurrence; and the second patient committed suicide still in complete remission 14 months after treatment (Table 2).

Because of their older age and the risk of fatal graft-v-host disease, most myeloma patients are candidates for autologous rather than allogeneic BMT. The tolerance of TBI/

HDM by a 63-year-old patient with primary unresponsive myeloma despite VAD and even HDM alone, with hematologic reconstitution from bone marrow harvested 1 year after HDM, suggests that this program can also be considered for patients initially requiring HDM alone because of marked marrow plasmacytosis. While the reinfusion of tumor cells is potentially detrimental to the use of autologous BMT, such an application seemed justified by the slower cell cycle kinetics of myeloma cells, in comparison with regenerating normal bone marrow.²¹ In addition, the terminally differentiated B cells that comprise this tumor have an unusually low in vitro, and perhaps in vivo, cloning efficiency.²² Thus, a selective growth advantage of normal hemopoietic cells over reinfused malignant plasma cells might be expected. While there is considerable interest in removing tumor cells from autologous marrow grafts by immunologic and/or cytotoxic means, such purging procedures must be specific for tumor cells so that hemopoietic engraftment is not compromised. The effectiveness of TBI/HDM, when supported by autologous BMT, provides a new approach to the therapy of patients with refractory myeloma and justifies further study in selected responding patients at high risk for relapse.

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CONCISE REPORT

Molecular Analysis of Relapse in Chronic Myeloid Leukemia After Allogeneic Bone Marrow Transplantation

By T.S. Ganesan, Gao Ling Min, J.M. Goldman, and B.D. Young

Four patients with Philadelphia (Ph⁺) positive chronic myeloid leukemia (CML) were studied before, after, and on relapse following allogeneic bone marrow transplantation (BMT). Southern analysis of DNA from cells collected before and at relapse after BMT was performed in order to investigate the origin of the leukemia at relapse. Using minisatellite probes we showed that the relapse occurred in cells of host origin in all four patients and this was confirmed with a Y chromosome specific probe in two male

patients who had a female donor. Furthermore, using two probes for the breakpoint cluster region (bcr) on chromosome 22, we showed that leukemic cells at relapse bore identical rearrangements to those in the disease at time of presentation of each patient. We conclude that relapse in all four patients is due to re-emergence of the original leukemic clone.

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A LLOGENEIC BONE MARROW transplantation (BMT) using HLA-identical siblings is currently the only approach that offers the possibility of cure for patients with chronic myeloid leukemia (CML). In a recent analysis performed for the International Bone Marrow Transplant Registry, the actuarial probability for survival in the chronic phase (CP) was 55% ± 5% and the actuarial probability of relapse was 19% ± 7% at 4 years.¹ When leukemia recurs, it is usually in cells of host origin but in two cases of CML the relapse occurred in cells of donor origin.^{2,3}

In this study molecular techniques have been used to examine the cellular origin of relapse in four patients with CML treated by allogeneic BMT. Minisatellite DNA probes and a probe specific for the Y chromosome were used to determine whether relapse occurred in donor or host cells.^{4,5} The relationship of the relapse to the leukemia at presentation was studied using probes specific for the breakpoint cluster region (bcr) on chromosome 22.⁶ The position of the breakpoint on chromosome 22 is different in each translocation in CML and thus the pattern of DNA rearrangement represents a unique clonal marker for each patient.

MATERIALS AND METHODS

Patients. Four patients were treated at the Hammersmith Hospital in London (Table 1). All risks of the treatment protocols were fully explained to patients, donors, and relatives. Informed consent was obtained from each patient in accordance with institutional guidelines. Each patient received chemo-radiotherapy followed by BMT from HLA identical siblings. Patients no. 2 and 4 had female donors. Three patients were in chronic phase and one patient was in blast transformation. The fourth patient presented with acute lymphoblastic leukemia (CALLA positive) in 1979 and achieved complete remission with conventional chemotherapy. In November 1980, he entered chronic phase of Philadelphia (Ph⁺) positive CML. He was transplanted with marrow from his sister in January 1982. He was well until February 1986, when he developed a granulocytic sarcoma as an extradural tumour at T6 and then relapsed with myeloid blast transformation in bone marrow and peripheral blood.

Cytogenetics. Cytogenetic analysis of bone marrow cells was attempted at 3-month intervals during the first year after transplantation and at 6-month intervals thereafter. Metaphases were examined by banding techniques.⁷

Chemotherapy. All four patients received cyclophosphamide 60 mg/kg body weight per day (on days -6 and -5) followed by total body irradiation (10 Gy or 12 Gy in five or six fractions, on days -4

to -2), delivered by a linear accelerator at a rate of 15 cGy per minute without lung shielding. One patient received busulphan (4 mg/kg/d orally on days -8 and -7) and the other three patients daunorubicin (60 mg/m² of surface area of day -7). Patients no. 2 and 3 received donor marrow depleted of T cells by in vitro incubation with the monoclonal antibody Campath-1 and complement. Donor marrow was transfused on day 0. Cyclosporine was administered for prevention of graft v host disease.⁸

Southern analysis. High molecular weight DNA was prepared from peripheral blood or frozen cells. It was digested with appropriate restriction enzymes and separated on a 0.8% agarose gel by the technique of Southern.⁹ Blotting was on to Hybond-N (Amersham, Amersham, UK), which was treated with UV light, prehybridized and hybridized at 43°C using 50% formamide as previously described.¹⁰ Radiolabeled probes were prepared by random primer extension to a specific activity of 1 to 3 × 10⁹ cpm/μg.¹¹ The filters were washed at 43°C in 0.1 × SSC (sodium chloride and sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate) and 0.1% SDS and were exposed at -70°C for one to four days. For the minisatellite probes, prehybridization and hybridization were carried out at 64°C and the filters washed at 64°C in 1 × SSC and 0.5% SDS.⁴

Probes

Minisatellite probes. The two probes used were 15.1.11.4 and 6.3 containing the core minisatellites from λ33.15 and λ33.6, respectively, cloned into M13mp19 and M13mp8 DNA, respectively.⁴

Y specific probe. As described previously, pHY2.1 was used as the Y specific probe.⁵

bcr probes. The 3' probe was a 0.6KB fragment subcloned from a commercially available probe (Oncogene Science, Mineola, NY). The 5' bcr probe was 0.45 KB fragment from the bcr cDNA clone as published previously from the K562 cell line.¹²

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Table 1. Clinical and Hematologic Features

Patient No.	Age/ Sex	Presentation		Duration Before BMT (mo)	Phase at BMT	Time to Relapse (mo)	Type of Relapse
		Spleen (cm)	WBC $\times 10^9/L$				
1	30/M	15	145	19	CP	21	BT-basophilic
2	34/M	15	557	60	BT	11	BT-myeloid
3	31/M	14	195	13	CP	11	CP
4	30/M	0	49	29	CP	53	BT-myeloid

Abbreviation: BT, blast transformation; CP, chronic phase.

RESULTS

The four patients in this study relapsed with Ph⁺ positive disease at various times after transplantation (Table 1). Three patients (no. 1, 2, 4) relapsed with acute phase disease without a preceding chronic phase, whereas one patient (no. 3) had Ph⁺ positive cells present 3 months before a chronic phase relapse.

Southern analysis of DNA from patients at presentation and at relapse following BMT with the minisatellite probes 15.1.11.4 and 6.3 showed in each case an identical pattern of restriction fragments (Fig 1).

Patients no. 2 and 4 had female donors. Using a Y specific probe pHY2.1, the intensity of the 2.1 kB restriction fragment was the same in these two patients before and after BMT (Fig 2), indicating that relapse occurred in cells bearing a Y chromosome.

Using the 3' bcr probe, the rearrangements observed before BMT were identical to the rearrangements at relapse in patients no. 2, 3, and 4. In no. 1, only the 5' bcr probe detected the rearrangement of the bcr gene and this was identical before and after BMT (Fig 3).

DISCUSSION

Two cases of leukemia recurring in donor cells after BMT for CML have been reported.^{2,2} The evidence that the relapse in donor cells was sex chromosome differences demonstrated in dividing cells. It is now possible to address this problem consistently in each patient using the DNA minisatellite probes.^{4,13} This minisatellite region was cloned from the intron of the human myoglobin gene and is highly polymorphic. Using a series of clones it is possible to derive a unique DNA "fingerprint" of each individual; this has been used in paternity testing and determination of twin zygosity.^{13,14} The probability of any two individuals having identical patterns is very low.^{4,13} It is therefore possible to use such an approach to distinguish host v donor origin of cells after relapse. Using two such clones as probes, 15.1.11.4 and 6.3, it was possible to show that the "fingerprints" for the original leukemias were identical to those of the relapsed leukemias in each patient, thus confirming the relapse to be in the hosts' cells. This was further confirmed by use of the Y specific probe pHY2.1 in two patients who had a female donor.⁵

G6PD analysis of Ph⁺ positive CML has suggested that the Ph⁺ translocation is a secondary event preceded by clonal expansion.¹⁵ It is therefore possible that the Ph⁺ positive relapse in host cells could be the result of a new genetic event. The molecular rearrangements in the bcr gene appear to be unique for each patient and hence a second independent translocation would be expected to result in a different

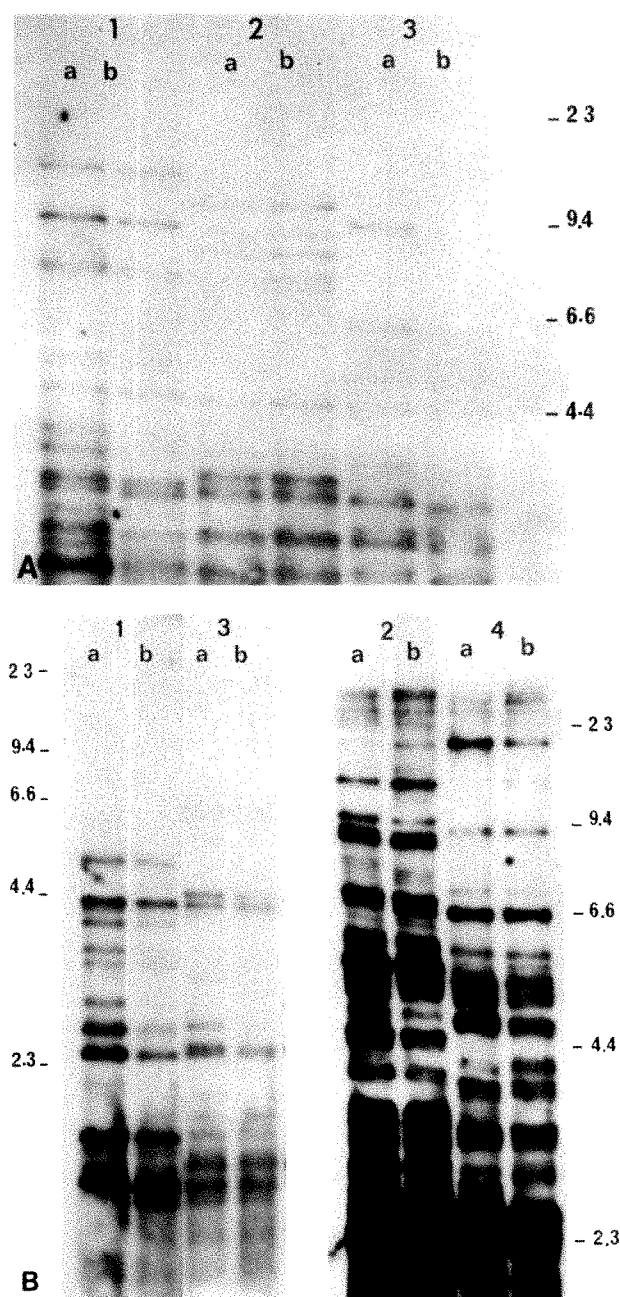


Fig 1. (A) Southern blot showing genomic DNA from patients no. 1, 2, and 3 digested with *HinF* and probed with 15.1.11.4; (B) patients no. 1, 2, 3, and 4 genomic DNA digested with *HinF*1, and probed with 6.3 a, pretransplant sample; b, posttransplant relapse sample.

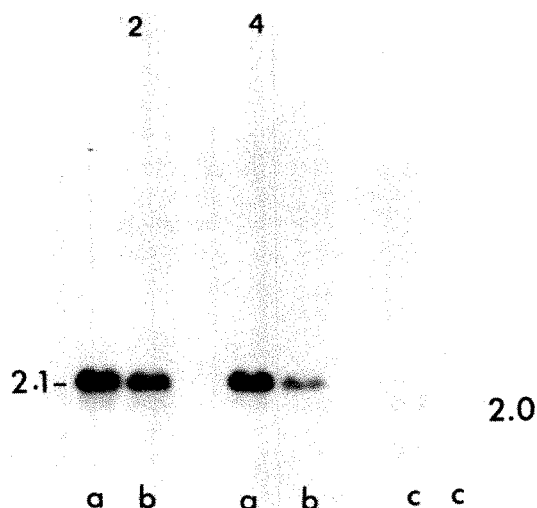


Fig 2. Southern blot showing DNA from patients no. 2 and 4 digested with *HaeIII* and probed with pHY2.1. a, pretransplant sample; b, posttransplant relapse sample; c, two normal female controls.

molecular pattern. Since the *bcr* gene rearrangements are identical before and after BMT in each of these four patients, we conclude that the original Ph⁺ positive clone is responsible for the relapse in each case. Furthermore, it is clear that in the evolution of the disease no further alteration to the *bcr* gene has taken place following the initial rearrangement.

In this preliminary study all four patients unequivocally demonstrated recurrence of the original clone even 4 years later as in patient no. 4. Further, as reported by Apperley et al,¹⁶ Ph⁺ positive metaphases can appear after BMT without hematologic relapse, only to disappear in time.¹⁶ Again this issue can be addressed by monitoring the rearrangement after BMT. Similar investigations have been carried out in acute lymphoblastic leukemia using immunoglobulin gene rearrangement as a clonal marker.¹⁷ In follicular lymphoma where t(14;18) is observed in the majority of cases, the breakpoints on chromosome 18 cluster within a transcriptionally active region.^{18,19} Using probes from this region the question of clonality can be addressed in patients with follicular lymphoma who have relapsed after any modality of therapy.

One explanation of recurrence of leukemia in donor cells after BMT is a transfection of viral or nonviral related oncogene to transplanted stem cells. An alternative hypothesis is that the stroma may be part of the leukemic clone and

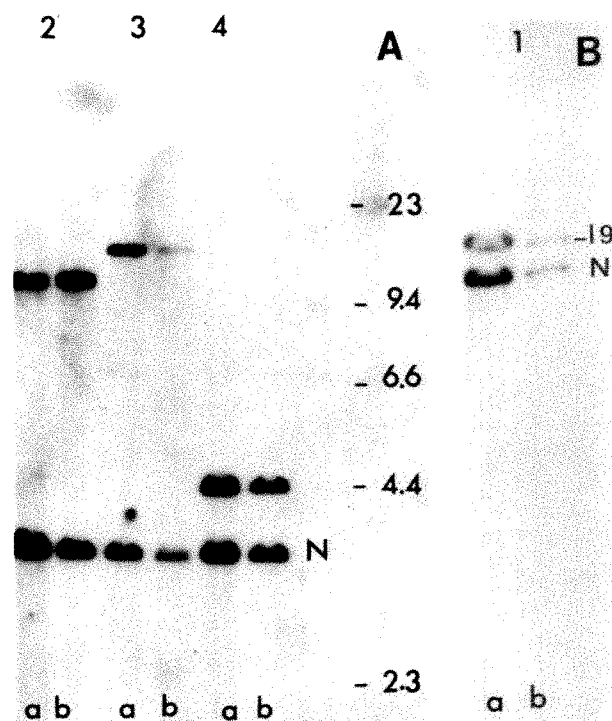


Fig 3. (A) Southern blot showing DNA from patients no. 2, 3, and 4 digested with *BamHI* and probed with 3' *bcr* probe; (B) Southern blot showing DNA from patient no. 1 digested with *BamHI* and probed with 5' *bcr* probe (N = germline). a, pretransplant sample; b, posttransplant relapse sample.

being radioresistant offers an abnormal milieu to donor cells. However, donor cell recurrent leukemia after BMT accounts for <5% of relapses.²⁰ Possibly normal donor stem cells are naturally resistant to the development of leukemia and there is a variable efficiency in transfection. It is now possible to characterize relapse after BMT particularly in CML with a combination of *bcr* and minisatellite probes. This study confirms that the relapsed leukemia is in the host cell in all the four patients. The recurrence in these four patients has been proven to be due to the original leukemic clone and the failure of the conditioning regimen and/or a graft v leukemia effect to eradicate it.

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CONCISE REPORT

Unrelated Donor Bone Marrow Transplantation Therapy for Chronic Myelogenous Leukemia

By Philip McGlave, Edward Scott, Norma Ramsay, Diane Arthur, Bruce Blazar, Jeffrey McCullough, and John Kersey

Eight patients received allogeneic bone marrow transplantation (BMT) as therapy for chronic myelogenous leukemia (CML) using marrow from unrelated donors. In all cases donors and recipients were HLA DR identical and had low MLC reactivity. In three cases recipients received marrow that was identical at the HLA A,B loci. In five cases HLA identity differed for one HLA A locus antigen. The unrelated donor search interval ranged from 2 to 28 months (median, 3 months). All recipients were prepared with a combination of cyclophosphamide, 60 mg/kg/d administered intravenously (IV) (days -6, -5) and with total body irradiation administered in 165 cGy fractions twice daily for four days (days -4, -3, -2, -1). Engraftment occurred in all cases (range, 18 to 48 days; median, 35 days), and return to a complete Philadelphia chromosome (Ph⁻) negative state was documented in six of eight cases. Moderate

or severe acute graft v host disease (GVHD) occurred in seven of eight cases, and extensive chronic GVHD in four of six evaluable recipients. A B cell lymphoproliferative disorder developed in one patient. Four recipients have died within 2 to 4 months of transplant. Four of eight patients survive at 11+ to 24+ months following transplantation (median, 15+ months) with normal peripheral blood counts and without evidence of leukemia. Current Karnofsky activity assessments are 90% or 100% in all survivors. Curative therapy of CML has been available only to the minority of patients eligible for sibling donor BMT. Unrelated donor BMT can be effective in the treatment of CML and may be particularly useful in this disorder since the prolonged stable phase of disease offers an opportunity to locate suitable donors.

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ALLOGENEIC BONE MARROW transplantation (BMT) can provide long-term disease-free survival when used as therapy for a variety of lethal disorders.¹ Unfortunately, the majority of otherwise eligible recipients lack a major histocompatibility complex (MHC) loci matched sibling donor. Bone marrow from unrelated donors matched or partially mismatched at the MHC loci may be used successfully in BMT therapy for severe combined immunodeficiency, aplastic anemia, and acute leukemia.²⁻⁷ Unrelated donor BMT may be particularly useful in the therapy of chronic myelogenous leukemia (CML) since marrow transplantation with sibling donors is known to be curative therapy for this disorder,⁸ and since the chronic phase of CML, with a median duration of over 3 years,⁹ may offer an adequate interval to locate a suitable unrelated donor. We present results of a clinical trial at the University of Minnesota studying the efficacy of unrelated donor BMT in the therapy of CML.

MATERIALS AND METHODS

Patient population. Eight consecutive Philadelphia chromosome (Ph⁺) positive CML patients found to have a suitable unrelated donor were transplanted between May 1985 and September 1986. All patients signed informed consent approved by the Committee on the Use of Human Subjects. Patient characteristics are described in Table 1. Ages ranged from 14 to 41 years (median, 25 years). Four patients (UPN 481, 587, 596, 634) were in chronic phase, two had features consistent with accelerated phase, including 14% bone marrow blasts in one case (UPN 534), and massive hepatosplenomegaly, 5% bone marrow blasts, and 10% bone marrow basophils in the other (UPN 570). One patient was in a second chronic phase after successful treatment of blast crisis with combination chemotherapy (UPN 480), and one patient was in a second blast crisis at transplantation (UPN 594). One patient (UPN 594) received a splenectomy before transplantation.

Preparation for transplant and graft v host disease prophylaxis. All recipients received cyclophosphamide, 60 mg/kg/d intravenously (IV) (days -6, -5) followed by 1,320 cGy total body irradiation in eight fractions of 165 cGy over four days (days -4, -3, -2, -1) administered with a 10 MV linear accelerator at a

dose rate of 8 to 10 cGy/min. Donor bone marrow that had not been T lymphocyte depleted was infused on day 0. All recipients received acute graft v host disease (GVHD) prophylaxis consisting of IV methotrexate, 15 mg/m² on day +1, 10 mg/m² on days +3, +6, +11 and weekly to day +100; IV antithymocyte globulin (ATG), 15 mg/kg on days +8, +10, +12, +14, +16, +18, and +20; oral prednisone, 40 mg/m² on day +8 to day +20.¹⁰

Unrelated donor search mechanism. The St Paul American Red Cross Blood Service maintains a bone marrow donor registry containing HLA A,B and DR identity of over 3,000 individuals.¹¹ All potential bone marrow donors have given informed consent before being enrolled in the registry. Potential donors with DR identity and with either HLA A,B antigen identity or with HLA nonidentity for one A antigen are located by computer search of the registry, contacted, and MLC typed. Suitably matched potential donors receive intensive education on the indications for transplant, the marrow donation procedure, and the potential risks of marrow donation. A community physician determines donor medical eligibility. Medically eligible donors are asked to sign informed consent forms approved by the Committee on the Use of Human Subjects at the University of Minnesota before bone marrow donation.

Donor/recipient MHC matching. In all eight cases reported here, marrow for transplant was obtained from unrelated individuals located through the registry operated by the St Paul American Red Cross Blood Service. HLA and MLC testing were carried out in the

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Table 1. Characteristics of Patients Undergoing Unrelated Donor BMT for Therapy of CML

Patient No.	Recipient Age/Sex	Disease Stage	Search Interval	HLA/DR Antigens*				Days to Graft	Days to Last RBC Transfusion†	Days to Last Platelet Transfusion†	Acute GVHD	Chronic GVHD	Survival (Karnofsky)	Cause of Death		
				A	B	DR										
480	14/M	2nd Chronic	28 mo	1	24	8	35	3	5	34	129	128	IV	Extensive	134 d	GVHD, pneumonia
				1	3	8	35	3	5							
481	22/F	Chronic	3 mo	1	23	8	44	3	7	40	61	71	—	—	736 + d (100%)	
				1	23	8	44	3	7							
534	16/F	Accelerated	2 mo	1	3	8	7	3	2	35	60	110	III	Extensive	551 + d (100%)	
				1	3	8	7	3	2							
570	16/M	Accelerated	7 mo	1	28	51	17	4	7	48	55	55	IV	Not evaluable	55 d	GVHD, pneumonia
				1	2	51	17	4	7							
587	41/M	Chronic	3 mo	2	3	7	w60	2	6	27	35	37	II	Extensive	377 + d (90%)	
				2		7	w60	2	6							
594	17/F	Blast	18 mo	1	2	w60	44	4	6	18	17	22	II	Extensive	127 d	GVHD, relapse
				1	34	w60	44	4	6							
596	30/F	Chronic	2 mo	1	2	8	44	3	4	40	102	79	III	—	338 + d (90%)	
				1	2	8	44	3	4							
634	28/M	Chronic	2 mo	1	11	7	w62	2	4	28	60	58	III	Not evaluable	60 d	GVHD, pneumonia
				1	11	7	w62	2	4							

*Recipient HLA/DR antigens represent genotype except in case of UPN 634 where insufficient data were available for genotyping. Donors were not genotyped. In each set the top line represents the recipient and the bottom line the donor.

†Interval to last transfusion associated with initial hospitalization. Two patients received additional transfusions in subsequent readmission for disseminated VZV (534) and for relapse (594).

Immunology Laboratory at the University of Minnesota. In each of the eight cases, one-way MLC performed in both directions demonstrated less than 15% reactivity when compared with unrelated, nonmatched, randomly chosen controls. All donor/recipient pairs were DR identical. In three cases, donor and recipient were identically matched at the HLA A and B loci, and in five cases donor and recipient were mismatched for one HLA A antigen (Table 1).

GVHD. Acute GVHD organ involvement was graded I to IV. Chronic GVHD was scored as "limited" or "extensive." Mild cutaneous acute GVHD (grade I) and limited chronic GVHD were treated with topical steroid cream. Patients who developed moderate or severe, acute (grade II to IV), or extensive chronic GVHD were treated with regimens containing systemic steroids and, in some cases, adjunctive therapy with ATG, cyclosporine, or azathioprine.

RESULTS

Identification of unrelated donors. Donor searches were submitted for 76 CML patients; unrelated donors meeting criteria described above were found in 15 cases. The median interval from submission of recipient HLA typing and location of a donor with suitable HLA identity and MLC nonreactivity in these 15 cases was 2 months (range, 1 to 28 months). In seven cases BMT using an unrelated donor was not performed due to death (two cases), medical complications (one case), unwillingness of a recipient to proceed (one case), and use of a father as donor (one case). One potential recipient with an acceptable unrelated donor has chosen to postpone BMT. Finally, objections of a spouse prevented one potential donor from participating in the protocol.

Engraftment and origin of engrafted cells. Engraftment, defined as peripheral blood leukocyte count $>1,000/\text{mm}^3$ for three consecutive days, occurred in all cases at a median of 35 days (range, 18 to 48 days). The interval from transplantation to last RBC transfusion associated with the acute posttransplant course ranged from 17 to 129 days (median, 60 days), and interval to last platelet transfusion ranged from 22 to 128 days (median, 65 days) (Table 1). In six cases studied by analysis of restriction fragment polymorphisms (RFLP),¹² DNA obtained from recipient peripheral blood or bone marrow samples revealed engraftment with cells of donor origin. In a seventh case (UPN 481), RFLP data were not available and cytogenetic analyses of bone marrow metaphases were not informative. This recipient, however, survives disease free at 24+ months. In the eighth case (UPN 570), although peripheral evidence of engraftment was demonstrated, RFLP analyses were not available, and no evaluable bone marrow metaphases were found before death at day +55.

Relapse. Evidence of relapse has occurred in one patient (UPN 594). This patient, a 17-year-old girl, was transplanted in a second blast crisis. Peripheral blood evidence of engraftment was documented at day +18. RFLP analysis performed on peripheral blood mononuclear cells at day +18 revealed that 100% of cells contained DNA polymorphisms identical to those of the donor. Cytogenetic analysis of bone marrow obtained at day +28, however, revealed Ph⁺ positive metaphases, and subsequent RFLP analysis revealed a population of peripheral blood mononuclear cells bearing DNA polymorphisms characteristic of recipient type. There was also morphologic evidence of recurrent CML in the periph-

eral blood and bone marrow. This patient died at day +127 with leukemia. Relapse has not been identified in any other recipient.

GVHD. Seven of eight recipients developed moderate or severe acute GVHD (Table 1). Evidence of extensive chronic GVHD occurred in four of six evaluable patients and included rash involving $>50\%$ of skin and serum bilirubin $>15\%$ (UPN 480), bilirubin $>15\%$ and involvement of oral mucosa (UPN 534), and rash involving $>50\%$ of skin (UPN 587, UPN 594).

B cell lymphoproliferative disorder. Right axillary node biopsy obtained from UPN 596 on day +76 revealed histologic and immunophenotypic abnormalities characteristic of B cell lymphoproliferative disorder.¹³ DNA analysis revealed multiple copies of the Epstein-Barr virus (EBV) genome. The patient was treated with IV acyclovir and subcutaneous alpha interferon. Clinical and CT examination revealed disappearance of hepatosplenomegaly and adenopathy over a 1-month period. The patient shows no evidence of abnormal lymphoproliferation or recurrent disease at 11+ months.

Causes of death. Four recipients died 55 to 134 days following BMT. Contributing causes of death included *Aspergillus sp* pneumonia (UPN 480), *Scopulariopsis sp* pneumonia (570), recurrent leukemia (594), and CMV pneumonitis (634). Moderate or severe acute, or extensive chronic GVHD occurred in all four cases. In the three cases complicated by fatal pneumonia, immunosuppressive therapy for GVHD was being administered when pneumonia developed.

Current status of survivors. Four patients survive at 11+ to 24+ months posttransplant. UPN 481 developed interstitial pneumonitis at day +30. Open lung biopsy revealed alveolar damage but no evidence of infection. Evidence of pneumonitis disappeared on oral prednisone therapy. Her course was otherwise relatively uncomplicated. The patient currently has no complaints, has normal exercise tolerance, and performs full time clerical work. Her Karnofsky assessment of activity is 100%. UPN 534 developed extensive chronic GVHD involving the liver and oropharynx that required prednisone, azathioprine, and cyclosporine therapy. She also developed a disseminated herpes zoster infection that was successfully treated with systemic acyclovir therapy. She has been taken off all immunosuppressive therapy, has a normal bilirubin, and has no physical evidence of GVHD. This patient is a full-time student with a Karnofsky activity assessment of 100%. UPN 587 developed extensive chronic GVHD of the skin and patchy interstitial pneumonitis at day +160. Open lung biopsy revealed lymphocytic infiltration of the bronchioles and no evidence of infection. All clinical evidence of active GVHD and pneumonitis disappeared on high-dose methylprednisone therapy. The patient does experience recurrent sinus infections that may be attributable to GVHD and is receiving oral prednisone, 30 mg every other day and azathioprine, 50 mg/d. This patient has resumed his work as an academician and has a Karnofsky activity assessment of 90%. As described above, the posttransplant course of UPN 596 was complicated by a B cell lymphoproliferative disorder that has resolved. She currently receives neither GVHD therapy nor therapy for

lymphoma, has resumed her job as a clerical worker, and complains only of mild fatigue. Her Karnofsky assessment is 90%.

DISCUSSION

We have tested the efficacy of unrelated donor BMT in the treatment of CML. To increase the likelihood of locating an unrelated donor, we searched for donor/recipient pairs in which there was low MLC reactivity, DR identity, and either HLA identity or a disparity of only one HLA A antigen.^{14,15}

Transplantation resulted in engraftment in all cases and disease-free survival ranging from 11+ to 24+ months in four CML recipients. The prolonged median time to engraftment (35 days) and prolonged median interval to cessation of RBC (60 days) and platelet support (65 days) may be related in part to the methotrexate and ATG containing acute GVHD prophylactic regimen, to the development of grade II to IV acute GVHD or to the development of other serious medical complications during the acute posttransplant course. The possibility exists, however, that delayed engraftment and prolonged transfusion requirements will be a feature of unrelated donor BMT, even when no discrepancy in immunologic typing between donor and recipient can be identified.

The relatively high incidence of acute GVHD seen in this series corresponds with a 63% projected incidence of grade II to IV acute GVHD at 3 months seen in a large series of CML patients undergoing matched sibling donor transplantation at this institution who received a similar preparative regimen and similar acute GVHD prophylaxis.¹⁶ Preliminary reports suggest that prophylaxis with combinations of methotrexate or prednisone and cyclosporine may provide a lower incidence of acute GVHD in sibling donor transplant therapy of chronic myelogenous leukemia or acute nonlymphocytic leukemia.^{17,18} Such an approach may be fruitful in the unrelated donor setting.

One patient undergoing unrelated donor BMT (UPN 596) developed a B cell lymphoproliferative disorder during the acute transplant course. Such abnormal lymphoproliferation, often associated with the presence of multiple copies of the EBV genome in lymphoid cells, has been seen following immunosuppression for organ and marrow transplantation. Lymphoproliferation is usually associated with T lympho-

cyte depletion of donor marrow, mismatched donor/recipient combinations, immunosuppressive therapy of GVHD, or a preexisting immunocompromised state.¹³ In this case the recipient received marrow from a donor who was HLA identical and MLC nonreactive in both directions. The importance of the unrelated status of the donor, despite MHC matching, in the development of lymphoproliferation is not known but is of obvious concern. Acute GVHD therapy with ATG may have contributed to an immunocompromised state predisposing to lymphoproliferation. The lymphoproliferative disorder resolved completely during alpha interferon therapy. We know of no published report of successful therapy for B cell lymphoproliferation with interferon and are not certain of interferon's role in the disappearance of the lymphoproliferation in this case.

All survivors were transplanted in chronic phase or with minimal evidence of acceleration (UPN 534), while all but one of four recipients (UPN 634) who died were transplanted with advanced disease. Better survival in the chronic phase group correlates with published results of sibling donor BMT therapy for CML; relapse in one patient transplanted in second blast crisis is also consistent with an observed higher incidence of disease recurrence in CML patients transplanted with advanced disease.^{8,16}

All four deaths occurred in patients who were HLA A antigen mismatched with their donors, while three survivors received transplants from HLA identical donors, and the fourth from a donor not expressing an HLA A antigen found in the recipient. The disproportionate number of deaths in the mismatched group cannot be ascribed to GVHD alone since the incidence of moderate, severe acute, or extensive chronic GVHD was high in both groups. Analyses of larger groups will be needed to determine the importance of HLA matching on outcome of unrelated donor BMT.

Curative therapy of CML has been available only to the minority of patients eligible for sibling donor BMT. Unrelated donor BMT can provide successful treatment of CML and should be considered when a suitable matched sibling donor is not available. Best results may occur when transplantation is performed before the onset of advanced disease. Further studies will be required to determine matching criteria and GVHD prophylaxis that provide the most satisfactory long-term disease-free survival.

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CONCISE REPORT

Molecular Genetic Rearrangements Distinguish Pre- and Post-Bone Marrow Transplantation Lymphoproliferative Processes

By Wesley J. Miller, Ralph S. Shapiro, Rogelio Gonzalez-Sarmiento, and John H. Kersey

Chronic myelocytic leukemia (CML) may display a lymphoproliferative phase (lymphoid blast crisis) that is generally of B cell phenotype. Since lymphoproliferative disorders may occur following bone marrow transplantation (BMT), it may be difficult to distinguish posttransplant relapse of CML lymphoid blast crisis from *de novo* lymphoproliferation. Lymphoid blast crisis cells from a patient with CML displayed immunoglobulin heavy chain gene ($C\mu$) rearrangement before BMT. Following BMT the patient developed a lymphoproliferative disorder involving multiple organs. Clonal rearrangement of $C\mu$ was demonstrated in

several involved tissues. The rearranged $C\mu$ restriction fragment was distinct from that displayed before BMT. Additionally, rearrangement of the breakpoint cluster region (*bcr*) was demonstrated in the pretransplant blast crisis sample, but not in the posttransplant lymphoproliferation samples, thus confirming that these lymphoproliferative disorders were distinct. Molecular genetic techniques offer powerful diagnostic tools for monitoring the course of patients with CML undergoing BMT.

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THE 9;22 CHROMOSOMAL translocation, which results in the Philadelphia chromosome typical of chronic myelocytic leukemia (CML), causes alterations (or rearrangements) of the breakpoint cluster region (*bcr*) located on the long arm of chromosome 22. In CML patients with the Philadelphia chromosome such rearrangements of the *bcr* can be demonstrated by the use of Southern blotting and genomic probes for this region.¹⁻³ CML frequently terminates in lymphoid blast crisis that is generally of B cell phenotype with expression of B lineage surface antigens and often with cytoplasmic immunoglobulin.⁴ In many cases of lymphoid blast crisis, immunoglobulin heavy chain rearrangement and in some cases light chain rearrangement can be demonstrated,^{5,6} indicating a monoclonal cell population. Following bone marrow transplantation (BMT), lymphoproliferative disorders may develop^{7,8} that can be difficult to distinguish from relapse of the primary disease, eg, CML. We report the use of immunoglobulin and *bcr* probes to distinguish posttransplant lymphoproliferation from relapse of lymphoid blast crisis in a patient with CML.

MATERIALS AND METHODS

Patient materials. The patient was a 12-year-old boy (UPN 588) with an established diagnosis of Philadelphia chromosome positive CML. He presented for transplant evaluation following lymphoid blast crisis. Induction chemotherapy returned his marrow morphology to a chronic phase appearance, and he underwent

further chemotherapy plus transplantation using haploidentical bone marrow from his mother; the bone marrow was depleted of mature T lymphocytes. Although he showed early engraftment, he became acutely ill with the abrupt onset of lymphadenopathy and fever on post-BMT day 30. Cervical node biopsy confirmed a diagnosis of lymphoproliferative process. In spite of attempted therapy he died 43 days after BMT. Pleural fluid, lung, lymph node, CSF, and bone marrow all showed involvement with lymphoblastoid cells. Procedures and studies were performed in accordance with institutional guidelines and with informed consent.

An enriched mononuclear cell population from peripheral blood during pre-BMT blast crisis was obtained by Ficoll-Hypaque centrifugation (45 minutes, 400 X G, room temperature). Cells were immediately frozen in RPMI with 10% dimethylsulfoxide and stored at -70°C. Immunophenotyping was done on fresh cells. Tissue specimens obtained at thoracotomy and at autopsy were frozen in liquid nitrogen.

Fresh peripheral blood cells and frozen tissues were studied for immunophenotype and extracted DNA was studied for gene rearrangements (as outlined below) for all samples.

Immunophenotyping. Cells were assessed for the presence of surface antigens associated with myeloid cells and with B and T lineage lymphocytes. For these studies cells were mixed with monoclonal antibody and incubated for 30 minutes at 4°C, washed and incubated with fluorescein-labeled anti-mouse antibody for an additional 30 minutes at 4°C. After three washes, cells were analyzed by Fluorescence Activated Flow Cytometry (Orthospectrum 3, Ortho Diagnostics Systems, Raritan, NJ, or FACS 4, Becton-Dickinson, Mountain View, CA). Appropriate controls using no monoclonal antibody were included to assess autofluorescence and control mouse ascites was used to determine background staining. Fluorescence profile was generated for each antibody tested, allowing percentage of antigen-positive cells to be determined. If more than 20% of cells tested were positive for a given antigen, these cells were considered to be positive for that antigen.

Monoclonal antibodies used in these studies included 35.1 (CD2), TA-1 (CD11a), which recognize T-cell associated surface antigens. Antibodies used to assess B lineage associated surface antigens included BA-1 (CD24), BA-2 (CD9) (found both on B cells and on granulocytes), and BA-3 (common acute lymphoblastic leukemia antigen, CD10). B1 (CD20) and B4 (CD19) were also used for some samples. MCS-2 recognizes a 150 kd glycoprotein (CDw13) present on myeloid and monocytic cells. This antibody does not bind to lymphoid cells of either B or T lineage. The OKM1 antibody was used to detect CD11 expression. Antibodies to HLA-DR and common leukocyte antigen (CLA) were also used. In addition, cells from the posttransplant specimens were stained with antibodies

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detecting immunoglobulin γ , α , and μ heavy chains and κ and λ light chains.

Extraction of DNA. High molecular weight DNA was extracted from pretransplant peripheral blood and posttransplant pleural fluid samples using the nuclei extraction technique described by Bakhshi et al.⁹ Briefly, cells were suspended in 5 mL of cold Fomace buffer, then 15 mL cold Fomace-Triton X-100 buffer was added followed by gentle mixing to resuspend cells. Cells were pelleted (1,500 rpm for ten minutes at 4°C), then resuspended in 25 mL Fomace-Triton buffer. Following pelleting, cells were washed twice in Fomace and then resuspended in 4.25 mL Fomace buffer, 0.2 mL 0.5 mol/L EDTA, proteinase K (2 mg/5 mL total solution). Sodium dodecyl sulfate (SDS) (10%) was added to final concentration of 1% (0.45 mL) and this mixture incubated at 37°C for three hours, following which 5 mol/L NaCl was added to final concentration of 0.5 mol/L NaCl. An equal volume of 1:1 mix of phenol and chloroform isoamyl alcohol (24:1) (CIAA) were added, mixed, and centrifuged (2,000 rpm for five minutes). The aqueous (top) layer was recovered and re-extracted, first using phenol and CIAA and then using CIAA. Cold (-20°C) absolute ethanol (2.5 vol) was added to the aqueous DNA solution. Following overnight incubation (-20°C) the DNA pellet was centrifuged (10,000 to 12,000 rpm for fifteen minutes at 4°C), ethanol discarded, the pellet dried, and resuspended in TE at a DNA concentration of approximately 1,000 $\mu\text{g/mL}$.

Solid tissues were first minced and homogenized in 5 mL TNE (.01 mol/L Tris, .01 mol/L NaCl, .001 mol/L EDTA) buffer. Proteinase K (100 $\mu\text{g/mL}$ final concentration) and 250 μL 10% SDS and 250 μL β -mercaptoethanol were added and the mixture incubated for three hours, 50°C. The mixture was then extracted three times (1 vol phenol, then 1 vol of phenol:CIAA mix, then 1 vol of CIAA). Sodium acetate ($\frac{1}{10}$ vol of 3M) was added to the aqueous DNA mixture before addition of 2.5 vol of cold absolute ethanol. The DNA was pelleted, dried and resuspended as outlined above.

Detection of gene rearrangement. Gene rearrangement studies used the gel transfer technique described by Southern.¹⁰ High molecular weight DNA was digested to completion using *Bam*HI restriction endonuclease. Restriction fragments were size separated by electrophoresis through 0.7% agarose (13 $\times$ 20 cm gel) at 40 V for 36 hours (*C μ*) or 0.8% agarose for 24 hours (*bcr*). Size separated DNA fragments were then transferred from the gel to nylon filter (Zetabind, AMF Cuno, Meriden, CT) in 20 $\times$ SSC. Following washing and prehybridization (with salmon sperm DNA) filters were hybridized in the presence of 50% formamide at 42°C for 24 hours using DNA probes labeled to high specific activity with ³²P-cytosine using random primer method.¹¹ Filters were washed for fifteen minutes in 2 $\times$ SSC, 0.1% SDS followed by a single wash with 0.1 $\times$ SSC, 0.1% SDS at 60°C. Filters were then exposed to x-ray film (Kodak, Rochester, NY) for autoradiography for twelve to 24 hours at -70°C.

Probes. Immunoglobulin gene heavy chain rearrangements were detected using a 1.3 kb DNA genomic probe for the heavy chain constant region (*C μ*) and κ -light chain rearrangements were studied with a 2.5 kb genomic constant region probe (*C κ*), both provided by S.J. Korsmeyer. Human *bcr* (PR-1) probe obtained from Oncogene (Mineola, NY) is a 1.2 kb *Hind*III-*Bgl*II restriction fragment of genomic DNA originally cloned from the breakpoint region of a patient with CML¹ and corresponds to an area in the intron between the third and fourth exons of the breakpoint cluster region.² In the formation of the Philadelphia chromosome, this portion of the *bcr* is usually translocated to chromosome 9.

Restriction fragment length polymorphisms (RFLP). RFLP were analyzed before and following BMT by Southern blotting as previously described.¹² For these studies, DNA was digested with *Taq*I and hybridized with probe pDP 34 (a gift from Dr David

Page). This test is routinely performed by the Molecular Diagnostics Laboratory for pre- and post-BMT samples.

RESULTS

Samples obtained before BMT at the time of lymphoid blast crisis and from the posttransplant episode showed lymphoblastic morphology. Cell marker studies showed that lymphoid cells obtained before transplant were HLA-DR, CD10 and CD9 positive, but were negative for CD2. These data were consistent with lymphoid blast crisis involving B-cell precursors. Posttransplant cells, however, were negative for all B cell, T cell, and most myeloid markers tested, although both HLA-DR and CLA were displayed. The posttransplant sample also did not display CD20 or CD19. Stains for cytoplasmic immunoglobulin showed cytoplasmic γ , α , and μ heavy chains in addition to κ and λ light chains, indicating a small polyclonal cell population in the lung tissue. The majority of cells from the lung tissue and all cells

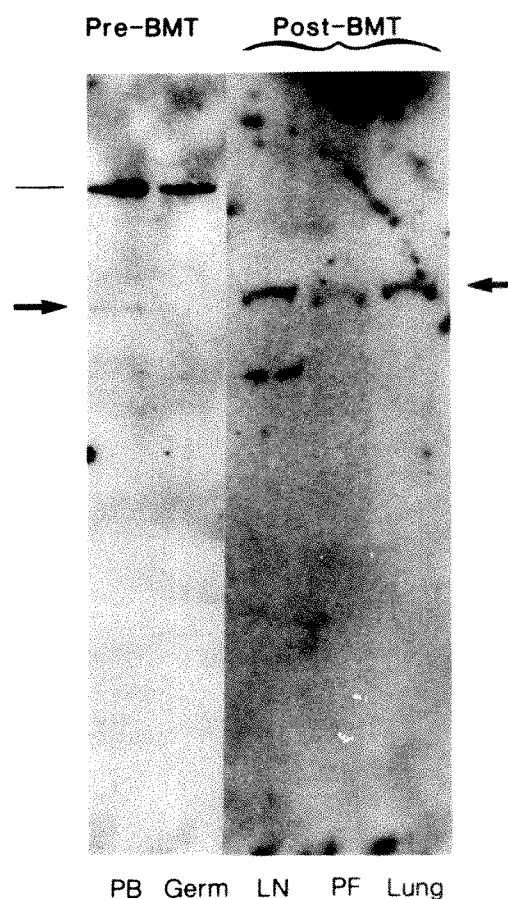


Fig 1. Southern blot analysis of immunoglobulin genes in pre- and post-BMT samples. Genomic DNA was digested with *Bam*HI and probed with a *C μ* immunoglobulin heavy chain constant region probe. The pre-BMT sample from peripheral blood (PB) during lymphoid blast crisis shows a rearranged *C μ* fragment of 5.4 kb (left arrow). Posttransplant samples that histologically represented lymphoma involving lymph node (LN), pleural fluid (PF), and lung samples all showed a 5.7 kb rearranged *C μ* fragment (right arrow). The germline DNA band position (lane Germ = placental DNA) is marked by a solid line on each side.

from the perihilar lymph node failed to stain for either cytoplasmic or surface immunoglobulin.

Molecular genetic studies confirmed the impression that the pre-transplant blast crisis cells were of B cell lineage, showing a clonal proliferation containing a 5.4 kb *Bam*HI $C\mu$ -hybridizing fragment (Fig 1). The posttransplant samples also showed rearrangement of the $C\mu$ region, resulting in a 5.7 kb *Bam*HI $C\mu$ restriction fragment (Fig 1) that was identical for the three samples studied (lymph node, pleural fluid, and lung), but which was different from the pretransplant blast crisis-associated rearrangement. The κ -light chain ($C\kappa$) was in the germline configuration in pre- and post-BMT samples. Analysis of the post-BMT lymphoproliferation for RFLP showed that these samples were entirely of donor cell origin. In studies of *bcr*, *Bam*HI-digested DNA from blast crisis showed rearrangement of *bcr*, whereas tissue from the three posttransplant samples show only germline *bcr* configuration (Fig 2).

DISCUSSION

This patient presented a somewhat difficult diagnostic challenge in that he developed a lymphoid malignancy during the very early post-BMT course. Cell marker studies

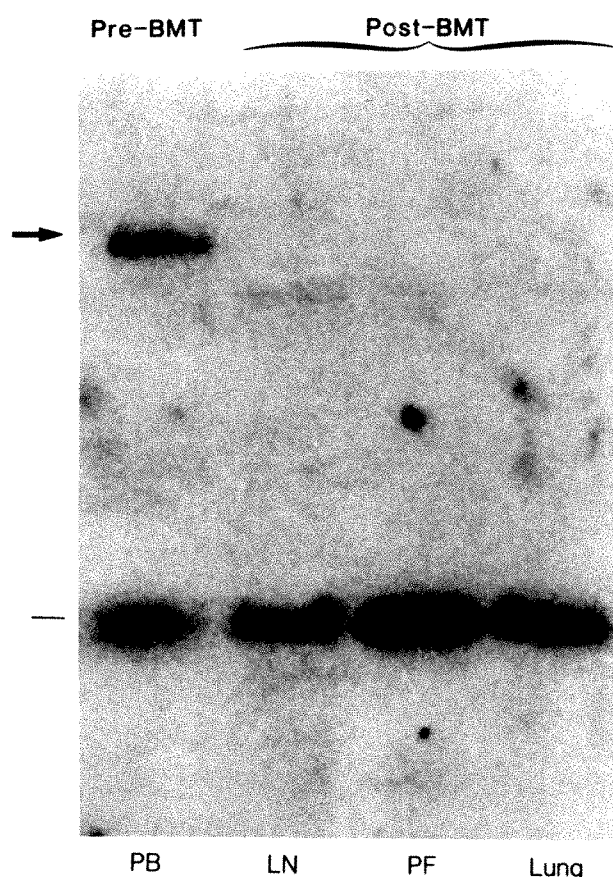


Fig 2. Southern blot analysis of *bcr* gene in pre- and post-BMT samples. Genomic DNA was digested with *Bam*HI and probed with a genomic probe for the *bcr* region of chromosome 22. The pre-BMT peripheral blood sample shows a rearranged fragment (arrow), while the post-BMT samples show only germline configuration of *bcr*. Germline band size is denoted by solid lines.

Table 1. Cell Marker Studies

Antigen	Pre-BMT	Post-BMT	
		Lymph Node	Lung*
CD2	—	—	—
CD9	+	—	—
CD10	+	—	—
CD11	+	NT	NT
CDw13	+	—	—
CD11a	+	—	—
CD19	NT	—	—
CD20	NT	—	—
CD24	—	—	—
HLA-DR	+	+	+
Cytoplasmic Ig			
γ	NT	—	+
μ	NT	—	+
α	NT	—	+
κ	NT	—	+
λ	NT	—	+

Abbreviation: NT, not tested.

*A polyclonal population staining for cytoplasmic Ig represents a small number of cells; a large percentage of cells show no staining for either surface or cytoplasmic immunoglobulin.

were inconclusive because of the heterogeneity in the various tissues studied. No B cell associated surface antigen was detected, although cytoplasmic γ , α , and μ heavy chains were detected as were κ and λ light chains. The nonstaining population of cells might represent a monoclonal population, but cell marker studies could not definitively demonstrate monoclonality. Immunoglobulin gene rearrangement has been demonstrated in both lymphoid blast crisis^{5,6} and in Epstein-Barr virus associated posttransplant lymphoproliferative disorders.^{7,13} The direct comparison of immunoglobulin gene rearrangements allowed us to say that this posttransplant lymphoid proliferation represented a different monoclonal disorder than that resulting in lymphoid blast crisis in this patient. In addition, these studies showed that the various sites of posttransplant lymphoproliferation involvement represented cells derived from a single clone as opposed to a multiclonal disorder arising simultaneously in different sites,⁷ since the $C\mu$ rearrangement was identical in all three samples. These data strongly suggest that this posttransplant lymphoproliferation represented a new B cell disorder unrelated to the pre-BMT lymphoid blast crisis. The presence of *bcr* rearrangement before but not after BMT supports this interpretation.

The post-BMT samples show deletion of one and rearrangement of one $C\mu$ allele, providing evidence of a monoclonal population of cells. The lack of germline band suggests that some polyclonal B-cells detected by markers may be dually rearranged for $C\mu$ and that cells retaining one germline allele may be present in numbers too small to detect. The large population of cells that represents the monoclonal lymphoproliferative disorder does not show cytoplasmic μ , suggesting that the detected Ig heavy chain rearrangement is nonproductive. The presence of germline kappa gene is also consistent with a non-productive Ig heavy chain rearrangement.

Molecular genetic techniques offer powerful tools for diagnosing and monitoring the course of a variety of hematological malignancies. Probing of the breakpoint cluster region in CML has now been demonstrated to be an effective means of following the course of CML patients posttransplant. A previous report by Bartram et al.¹⁴ showed that this method may predict CML relapse following BMT before the detection of the Philadelphia chromosome by standard cytogenetics. We now have described a case in which possible

post-BMT relapse was ruled out by the presence of *bcr* rearrangement before but not following bone marrow transplantation and on the basis of unique immunoglobulin gene rearrangements. As more effective and specific therapies become available, it will be extremely important to distinguish between new malignancies and those which represent posttransplant relapses. Such studies are of value in interpreting the results of BMT.

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CORRESPONDENCE

PROPHYLACTIC HEPARIN IN APL

To the Editor:

In a recent article, Goldberg and colleagues¹ presented the Brigham and Women's Hospital and Dana Farber Cancer Institute's experience in managing acute promyelocytic leukemia (APL) without heparin. They also analyzed historical data and concluded "that the coagulopathy associated with APL can be successfully managed with intensive chemotherapy and blood product support" and that "the necessity for the inclusion of prophylactic heparin therapy into the remission induction regimens of all patients with APL has not been proven."

Although the benefit of prophylactic heparin in APL is unproven, the thrust of the article made a case against the use of heparin and by its title ("Is heparin administration necessary during induction chemotherapy for patients with acute promyelocytic leukemia?") suggested the exclusion of heparin in the management of these patients. Because of flaws in this study and in the authors' analysis of previous studies, however, one can make no conclusion about the use of heparin in patients with APL based on this article.

In presenting their own data, the authors demonstrate a complete remission rate comparable to that of series that have included heparin in conjunction with anthracycline-based chemotherapy.^{2,3} Because the data presented did not include coagulation parameters at the time of entry nor an indication of bleeding status, however, the results may reflect a population of patients with a lesser bleeding diathesis. The paucity of data makes a meaningful conclusion about the comparability of this series to other series in the literature impossible.

Also of concern is the authors' use of historical studies to analyze the cumulative experience with and without heparin and their application of a statistical method to validate their conclusions. Based on their own experience combined with eight series of patients treated during different decades with different protocols and variable ancillary support, the authors concluded that no statistically proven benefit for heparin was evident. No statistical test could accurately be applied to such divergent studies.

Furthermore, the authors' extraction of data from the eight references was flawed (Table 1). They stated that their comparison was limited to anthracycline-based protocols, yet 13 of 60 patients treated by Kantarjian and colleagues⁴ did not receive anthracyclines. Four additional hemorrhagic deaths among patients treated without heparin were not included in the analysis when that stipulation was applied to another study.⁵

Furthermore, nine deaths included among the hemorrhagic deaths of patients treated with heparin occurred during aplasia, by which time the heparin should have been discontinued. The data from Cunningham and co-workers³ were incorrectly reported. Correcting

Table 1. Effect of Heparin on Incidence of Hemorrhagic Deaths in Adults with APL During Induction Chemotherapy

Reference	With Heparin		Without Heparin	
	n	Hemorrhagic Deaths	n	Hemorrhagic Deaths
Drapkin et al	9	2 (0)*	15 (8)	9 (5)
Ruggero et al	0	—	13	4
Daly et al	10	1	5	1
Collins et al	7	1	0	—
Bernard et al	9	4	35	9
Cordonnier et al	57	6 (15)	0	—
Cunningham et al	50 (54)	6 (7)	3	0 (1)
Kantarjian et al	31	6	28	9
Goldberg et al	2	0	25	4
Total	175	26 (15%)	124	36 (29%)

Data as extracted from Goldberg et al.¹

*Numbers in parentheses represent data as reported by Goldberg.¹

Total data were determined by the authors' review of the cited reports.

for these discrepant values (Table 1) gives a hemorrhage-related death rate of 15% in patients treated with heparin and of 29% in patients managed without heparin during induction therapy.

Although we agree that no definite evidence shows heparin to be a necessary component in the treatment of APL, heparin clearly has not been shown to be deleterious in this setting, despite the authors' legitimate concern regarding the administration of heparin to "thrombocytopenic, critically ill patients." Confirmation of risk factors predicting for a hemorrhagic diathesis, such as absolute blast count⁴ or α 2-plasmin inhibitor level,⁶ as has recently been suggested, could identify those patients most in need of treatment of their coagulopathy with heparin and/or epsilon-aminocaproic acid.⁶

We agree with the authors' final conclusion that a prospective randomized trial is required. Only by this type of analysis can the optimal management of acute promyelocytic leukemia be determined.

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To the Editor:

We are pleased that Drs Venook, Shuman, and Corash concur with our conclusion¹ that the benefit of prophylactic heparin in the management of acute promyelocytic leukemia (APL) is presently unproven. Furthermore, we are glad that they share our stated concerns over the use of historical controls and that they concur with our final recommendation that a prospective, randomized trial would be required to determine the optimal means of providing supportive care for patients with APL. We take serious exception, however, to the implication that our analysis of previous studies was basically flawed. The data attributed to Cunningham and colleagues² were based on updated figures kindly provided to us in a personal communication; perhaps the data should have been referenced as such. With regard to the report by Kantarjian and co-workers,³ we stand technically corrected in that 13 of the 60 patients did not receive an anthracycline but rather were treated with amsacrine. Our purpose in attempting to limit our analysis to anthracycline-based regimens was to focus our analysis on somewhat more contemporary, intensive chemotherapeutic regimens in an effort to avoid potentially problematic comparisons with older historical controls. The inclusion of the amsacrine-based regimens of Kantarjian and colleagues³ conforms with this reasoning since they are intensive regimens and have been shown to have efficacy similar to that of anthracycline-based regimens. We wanted to exclude less comparable, less intensive regimens such as the drug combination given to seven of the patients of Drapkin and colleagues⁴ between 1970 and 1973 (included in Table 1 of Venook et al). Even taking into account these trivial changes in the data of Cunningham and colleagues² and Drabkin and associates,⁴ however, and including all of the data from Kantarjian and co-workers,³ we still obtain a hemorrhagic death rate of 20% (35 of 175) in patients treated with heparin and 29% (36 of 124) in patients not receiving heparin. This is not significantly different from the hemorrhage-related death rate of 19% (34 of 179) in heparin-treated patients and 28% (33 of 117) in patients not receiving heparin reported in our article.¹

The only significant change in interpretation of previous studies which Venook and colleagues apparently wish to make is in the study

of Cordonnier and colleagues.⁵ We are uncertain on what basis they make their contention that we misrepresented the results obtained by Cordonnier and colleagues.⁵ Cordonnier and colleagues⁵ clearly state that 15 of their 57 heparin-treated patients suffered hemorrhagic deaths. Nine of these deaths did occur during aplasia, and heparin treatment or disseminated intravascular coagulation (DIC) was clearly implicated in at least 5 of these 9 deaths. It is the analysis of Venook and colleagues that is flawed when they discount these 9 deaths because they feel that "heparin should have been discontinued." The results of Cordonnier and colleagues⁵ must stand on their own and should not be changed to reflect what Venook and colleagues believe "should have been."

It is also incorrect that coagulation parameters were not included in our report. Table 2 in our article clearly shows that 23 of 27 (85%) of our patients had DIC initially or shortly after beginning induction therapy. This is comparable to many previously reported series and does not appear to reflect a population of patients with a lesser bleeding diathesis.

Finally, the main point of our article was to make clear that although heparin is accepted as a standard method of practice, it has not been shown to be a necessary component in the treatment of APL. In our experience, the coagulopathy associated with APL can be successfully managed with intensive chemotherapy and blood product support without routine use of heparin. We believe it is contrary to the usual practice of medicine to adopt a potentially toxic therapy prior to demonstrating its benefit.

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DISTRIBUTION OF TRANSFERRIN RECEPTORS IN THE CELLULAR COMPONENTS OF THE LIVER

To the Editor:

The recent work by Vogel et al,¹ demonstrating the presence of transferrin receptors in nonparenchymal cells of the liver, is interesting and very much in line with our own published work.

Regrettably, however, this recent work is subject to the same criticism that the earlier works by Young and Aisen^{2,3} were. The basic assumption in kinetic studies of receptor-ligand interaction using radiolabeled ligand is that the cell population under study is homogeneous. This is because in all calculations the denominator is the number of cells. Clearly, if a proportion of cells have a certain type of receptor and others do not, calculation of affinity, maximum

binding, and number of receptors per cell would be erroneous. The earlier work by Young and Aisen^{2,3} was subject to this criticism because they used crude liver cell suspensions (a mixture of parenchymal and nonparenchymal cells) and attributed all the binding to parenchymal cells. Although Vogel et al fractionated parenchymal cells, they treat nonparenchymal cells as if they were a homogeneous cell population; they are not. They contain both Kupffer cells and endothelial cells. The latter cells, but not the former as we have demonstrated,^{4,6} possess transferrin receptors. The heterogeneity of this cell population invalidates results obtained by the application of the radiolabeled ligand binding assay.

Similarly, morphologic studies are subject to criticism. The use of the immunoperoxidase technique on a tissue that has cellular components (hepatocytes and Kupffer cells) that contain significant endogenous peroxidase is not an optimal approach. The authors state¹ that the endogenous peroxidase was blocked by incubation with 1.5% H₂O₂-PBS for 30 minutes. However, this type of inhibition is at best partial and incomplete. The use of immunoperoxidase on a tissue that is devoid of endogenous peroxidase may be acceptable. In the liver, however, it would be preferable to use other immunoenzyme techniques and even more preferable to work at the electron microscopic level to provide better resolution.

The most distressing part of the study of Vogel et al, however, is the discrepancy between the kinetic data provided by them and those provided in previous papers by Young and Aisen.^{2,3} The latter investigators reported the affinity constant of hepatocyte receptors to be in the range of $1 \times 10^7 \text{ mol}^{-1}$. This is the same range that we also have obtained both for hepatocyte and liver endothelium.⁷ Vogel et al provide us with affinity data that are higher by at least one order of magnitude. The authors did not provide an explanation for this discrepancy in two studies from their own group.

The affinity data for hepatocytes and nonparenchymal cells are given respectively in the text as 2.6 and $3.3 \times 10^8 \text{ mol}^{-1}$, while in Fig 7 they are given as 3.4 and 5.1 . Also the unit of affinity is mol^{-1} , which means $1/\text{mol}$. What kind of unit is $1/\text{mol}^{-1}$?

It is curious, however, that the affinity provided by Vogel et al ($K_a 2.6 \pm 0.8 \times 10^8 \text{ mol}^{-1}$) is closer to the affinity of asialo-glycoprotein receptors ($K_a 2.4 \times 10^9 \text{ mol}^{-1}$) than transferrin receptors^{8,9} (K_a

$1.05 \times 10^7 \text{ mol}^{-1}$ according to their own data³). This would suggest that at least part of the transferrin binding to hepatocytes might have occurred via galactosyl receptors. Since the authors use anti-transferrin antibody (rather than antitransferrin receptor antibody) to detect surface bound ligand, the detection method cannot differentiate between the ligand bound through transferrin receptors and galactosyl receptors.

Transferrin is a glycoprotein whose glycan chain terminates in sialic acids, with the penultimate residues being galactosyl.¹⁰ In our hands a significant proportion of transferrin is partially or totally desialated. This has also been the experience of others^{11,12} who have found that in certain states the proportion may increase. In our laboratory, we routinely pass our transferrin preparations through an RCA₁₂₀ agarose affinity column to remove the desialated fraction. This column retains desialated molecules terminating in galactosyl residues, permitting only sialated molecules to pass. This would eliminate the binding of ligand via asialoglycoprotein receptors. It would be advisable if these authors similarly fractionated their transferrin preparations to remove asialotransferrin. This may overcome the discrepancy between their two studies.

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To the Editor:

Tavassoli and Harjes state that there is a discrepancy in the affinity of binding of ¹²⁵I-transferrin to its receptor in preparations of unfractionated rat liver cells^{1,2} as compared with the values reported in the present paper³ in which preparations were fractionated to yield parenchymal and nonparenchymal cells. In the first two studies,^{1,2} the ligand used was the major isotransferrin of rat serum and affinities of the order of 10^7 l.mol^{-1} were reported. In our recent paper for the reasons discussed we used human transferrin and found that it bound consistently with higher affinities of the order of 10^8 l.mol^{-1} . Because of uncertainties over the nature and location of the transferrin receptor among liver cell populations we were asked to perform additional experiments to compare the binding of human and rat transferrins with a cell type that unequivocally expresses the rat transferrin receptor, the rat reticulocyte. Again we found that human transferrin bound with a higher affinity than did rat transferrin (Fig 1, ref 3). In this figure and in Fig 7 the unit of affinity was

inadvertently given as $1/\text{mol}^{-1}$; $1.\text{mol}^{-1}$ is correct. In agreement with these results the quantity of human diferric transferrin required to inhibit by 50% the binding of iodinated human transferrin to rat reticulocytes was at least tenfold less than the amount of rat diferric transferrin required. Interestingly, Rudolph et al⁴ recently found in similar competition experiments that the quantity of human diferric transferrin required to inhibit by 50% the binding of rat ¹²⁵I-transferrin to rat reticulocytes was at least five times less than the amount of diferric rat transferrin required.

Concern has also been voiced about the values given for affinity constants. Figure 7 (ref 3) shows the data derived from the single equilibrium binding experiment given in Fig 6. In this experiment, Scatchard analysis yielded affinities of 3.4 and $5.1 \times 10^8 \text{ l.mol}^{-1}$ for the binding of human diferric transferrin to parenchymal and nonparenchymal cells, respectively. The values in the text are mean ($\pm$ SD) values of four separate binding experiments that gave affinities of $2.6 \pm 0.8 \times 10^8 \text{ l.mol}^{-1}$ and $3.3 \pm 1.9 \times 10^8 \text{ l.mol}^{-1}$ for parenchymal and nonparenchymal cells, respectively.

We do not agree that transferrin receptors are restricted to the endothelial cells and have shown using an immune-adherence technique that both Kupffer cells and endothelial cells express sufficient transferrin receptors to adhere in the presence of diferric transferrin to antitransferrin antibodies immobilized on Staphylococcus A-coated petri dishes.⁵

With regard to the binding of asialo-transferrin to hepatocytes we have previously shown that rat asialo-transferrin binds to the hepatic asialo-glycoprotein receptor with an affinity of $1.4 \times 10^6 \text{ l.mol}^{-1}$ which, as we have already stressed, is much lower than the affinities found for other asialoglycoproteins,⁶ for example, asialoorosomucoid.⁷ We speculated that this low binding constant may reflect the relatively low carbohydrate content of the major rat isotransferrin, which possesses only one biantennary glycan chain, whereas orosomucoid has five side chains of the complex type (discussion in ref 6). Thus the presence of contaminating asialo-transferrin should lower the affinity constant and not, as suggested by Tavassoli and Harjes, increase it.

Finally, the immunoperoxidase technique has, in our hands, proved satisfactory, especially when accompanied by careful control experiments.

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REJECTION OF ALLOGENEIC BONE MARROW CELLS

To the Editor:

The general principles governing allogeneic bone marrow rejection remain controversial. Initial results were compatible with lymphocytes (presumably T) as effector cells, similar to the rejection of other solid tissues.¹ Subsequently, Cudkovic and others later postulated a unique rejection mechanism for bone marrow cells that would inactivate allogeneic cells within 72 hours after injection.² This effector mechanism has been called allogeneic resistance and would be largely T cell independent, radioresistant, and responsive to antimacrophage or anti-NK (natural killer) cell measures. An antibody dependent cellular cytotoxicity mechanism has also been suggested.³ Other investigators have provided evidence that allogeneic resistance does not offer an adequate explanation for bone marrow rejection, mainly because the assays used to explore allogeneic resistance are spleen assays, which may not be predictive for what happens to hemopoiesis in the bone marrow.⁴⁻⁶ As a consequence, one can maintain that rejection of bone marrow cells does not differ from the rejection of other allogeneic tissues.

New arguments for a unique mechanism for allogeneic bone marrow rejection were published recently by Raff et al and Butturini et al, in studies of dogs and humans.^{7,8} They found that some peripheral blood lymphocytes survive high doses of total body irradiation (TBI) and argue that these must be uniquely radioresistant cells that are responsible for bone marrow rejection. However, their results are not surprising and are predicted by generally accepted radiobiology models. If one assumes that approximately 1.5% of the body weight is due to lymphocytes,⁹ and that a multitarget model with an extrapolation number (n) of 1.25 and a D_{01} of 1.25 Gy applies to lymphocytes under the conditions of their TBI procedures, (surviving fraction = $1 - (1 - e^{-D/D_{01}})^n$, approximately

one hundred million and two hundred million lymphocytes would survive in standard humans and dogs, respectively. Such lymphocytes would not be radioresistant, as cell survival or cell kill by radiation is a random process. Radiation resistance (spontaneous or induced by radiation) is a rare phenomenon. It could only be proven for the experiments under discussion by obtaining dose/effect curves for cells obtained before and after TBI. The technology to perform such experiments for human lymphocytes remains to be developed. The unique phenotype of lymphocytes surviving TBI that was found by Raff et al⁷ might be a reflection of the waves of cell depletion and repopulation that occur in that setting. In the absence of specificity controls (host/donor origin; activity against donor) the presence of a rare cell population immediately after TBI cannot be taken as proof for unique effector cells in bone marrow rejection.

In mice, only 50,000 lymphocytes were required to reject rat bone marrow cells after TBI.¹ Detailed mechanistic studies of bone marrow rejection are difficult in design and execution, but T lymphocytes of normal radiosensitivity remain prime candidates for effector cells in allogeneic bone marrow rejection. Rejection models based on allogeneic resistance concepts or on radioresistant effector cells are not supported by the currently available information. The subtype and number of lymphocytes required to reject an allogeneic bone marrow transplant has not been determined. A better understanding of bone marrow allograft rejection will be extremely helpful in the design of future TBI protocols with a higher therapeutic ratio than the currently employed TBI procedures.

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To the Editor:

Dr Vriesendorp indicates that it is not surprising that lymphocytes survive high-dose radiation and this might not be related to inherent radioresistance; this is obvious. The occasional survival of recipient lymphocytes after high-dose chemoradiotherapy is readily predicted. In some cases of graft rejection, persistence of recipient immunocompetent cells has been reported.

The object of our study was to demonstrate that clonable immunocompetent T cells persist after intensive conditioning for bone marrow transplantation in a relatively high proportion of patients. Since these cells can mediate graft rejection, they must be consid-

ered in the dynamics of HVG/GVH reactions. Other variables include the number of donor T cells in the graft and the degree of genetic disparity between donor and recipient. Parenthetically, we never used the term "radio-resistance" nor postulated mechanisms of survival of recipient T cells.

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ANNOUNCEMENTS

HOUSING ARRANGEMENTS FOR THE 1987 ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY

Multiple hotels will be utilized in Washington, DC to house convention registrants for the annual meeting of the American Society of Hematology to be held December 5-8, 1987. Accommodations will be handled through the ASH Housing Bureau in Washington. To obtain your hotel reservation form, please write to the ASH Registration Supervisor, c/o SLACK Inc. 6900 Grove Rd, Thorofare, NJ 08086-9447.

WILMOT CANCER RESEARCH FELLOWSHIPS

The University of Rochester School of Medicine offers research training to physicians who have completed at least 1 year of residency or research experience. Fellowships will begin July 1, 1988. Opportunities exist in any discipline relevant to the cause, diagnosis, treatment, or prevention of cancer, including endocrinology, experimental therapeutics, genetics, hematology, immunology, microbiology, molecular and cellular biology, pathology, and radiation oncology.

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For further information, contact: Wilmot Cancer Research Fellowship Program, Medical Center Box 601, University of Rochester School of Medicine & Dentistry, 601 Elmwood Ave, Rochester, NY 14642.

**BLOOD BANK DIRECTORS' TRAINING PROGRAM
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The New York Blood Center offers a postdoctoral training program for qualified physicians interested in a career in blood transfusion services. It is anticipated that persons completing the program will serve as fulltime directors or associate directors of regional blood programs or hospital blood banks, research scientists, medical school faculty members, or members of government regulatory advisory agencies. In the initial year, the training Fellow receives instruction in immunohematology, immunogenetics, tissue typing, cryobiology, virology, blood component preparation, and management in the Greater New York Blood Program. In addition, each Fellow is assigned to a participating, university-affiliated hospital blood bank in the New York area, for training in clinical transfusion therapy, coagulation studies, and blood bank technology. A second year can be arranged to suit individual requirements for more intensive training in one or more areas. The program is accredited by the Accreditation Council for Graduate Medical Education; the entire year is acceptable towards meeting eligibility requirements for the subspecialty examination in blood banking of the American Board of Pathology.

Requirements: (1) An MD degree with a minimum of 2 years postdoctoral training in clinical medicine or pathology; (2) a New York medical license by the beginning of the fellowship year; and (3) US citizenship, permanent residence, or exchange visitor status.

Stipend: Comparable to the fifth year postgraduate training stipends in the New York area.

Applications: Applications for the program beginning July 1, 1988 must be received before December 15, 1987.

For further information, contact: Robert F. Reiss, MD, Director of Clinical Services, The New York Blood Center, 310 E 67th St, New York, NY 10021.

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APLASTIC ANEMIA FOUNDATION OF AMERICA (AAFA) RESEARCH FELLOWSHIP AWARD

The AAFA has established a Research Fellowship Award in order to encourage young researchers and to recognize their contributions to research related to aplastic anemia. In January 1988, an award of \$25,000 will be made for a research fellowship to begin July 1988. The deadline for completion of the application package is November 15, 1987.

For information concerning eligibility criteria and application forms, write: Research Fellowship Award, AAFA, PO Box 22689, Baltimore, MD 21203.

LASERS IN MEDICINE: Expanding Clinical Applications

**October 10, 1987
Alta Bates Hospital Auditorium
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This conference is approved for 7.5 hours Category 1 credit by the CMA; fees are \$65.00 for physicians and \$30.00 for nurses and residents. The conference will run from 8:00 AM to 4:00 PM.

For more information, contact: Mary Grim, Medical Education Coordinator, (415) 540-1420.

COAGULATION RESEARCH AND TEST DEVELOPMENT

The University of Nebraska Medical Center is seeking a PhD (Assistant Professor, tenure track) with expertise in coagulation research and clinical test development as technical director of a clinical coagulation laboratory. UNMC is a regional referral center for hemophilia and thrombotic diseases.

Send CV and three letters of recommendation by 11/30/87 to Dennis Weisenburger, MD, Department of Pathology and Microbiology, UNMC, 42nd and Dewey Ave, Omaha, NE 68105.

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ERRATA

In the article by Chabanel, Reinhart, and Chien entitled "Increased Resistance to Membrane Deformation of Shape-Transformed Human Red Blood Cells," which appeared in the March 1987 issue (Vol 69, pp 739-743), there was an error on page 740 (column 2, line 7): $1/0.245$ K; the value of 0.245 should read $1/2.45$ K; the value of 2.45.

In the article by Sundeen et al entitled "Rearranged Antigen Receptor Genes in Hodgkin's Disease," which appeared in the July 1987 issue (Volume 70, pp 96-103), there were a number of errors in Figs 2-7, and in the legend for Fig 3. The correct figures and legends appear below.

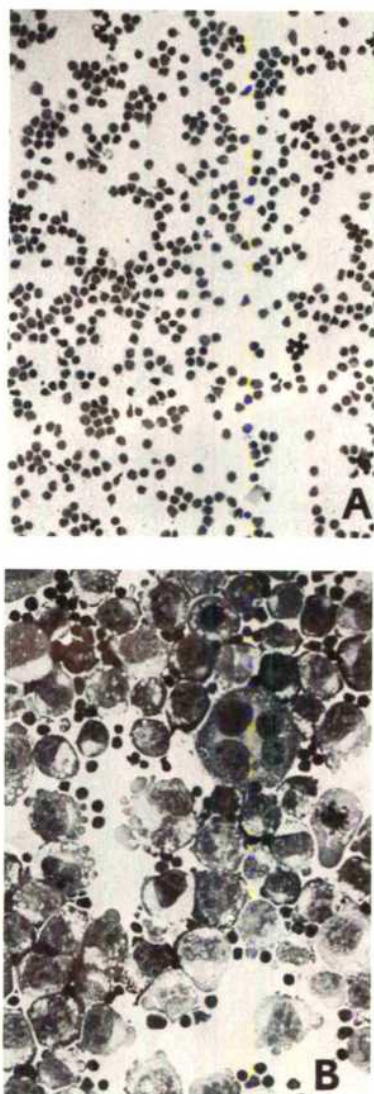


Fig 2. Density gradient separation of Reed-Sternberg cells and variants (case 5). A cell suspension of an involved lymph node, which contained 0.04% Reed-Sternberg cells and variants, was fractionated by centrifugation through a discontinuous Percoll gradient. (A) Lymphocytes (>99%) were recovered from the pellet (density >1.069 g/mL) (Original magnification $\times 400$; current magnification $\times 263$; Wright's stain). (B) Reed-Sternberg cells and their mononuclear and multinuclear variants were highly enriched in the interface at density 1.069 g/mL (original magnification $\times 400$; current magnification $\times 270$, Wright's stain).

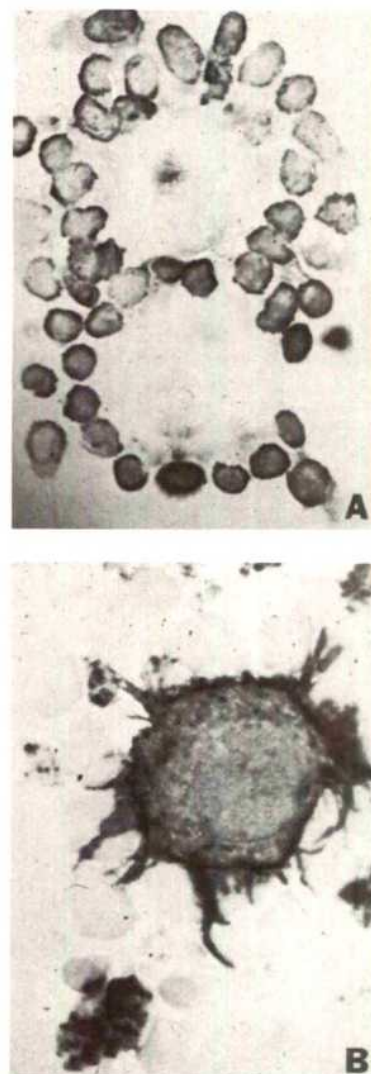


Fig 3. Immunostaining of cytocentrifuge preparations of Reed-Sternberg cell-enriched fraction (case 5). (A) Spontaneous adherence to T cells to mononuclear variants of Reed-Sternberg cells. Reed-Sternberg cells and variants did not react with the anti-T cell antibody Leu-4 (Leu-4, ABC; original magnification $\times 1,000$ current magnification $\times 650$). (B) Leu-M1 staining of a mononuclear variant of a Reed-Sternberg cell reveals elaborate dendritic processes that engulf adherent T cells (Leu-M1, ABC; original magnification $\times 1,000$, current magnification $\times 650$).



Fig 4. Southern analysis of immunoglobulin κ genes from unfractionated Hodgkin's tissues. Filter hybridization of *Bam*HI digests of genomic DNA obtained from cases 1 through 4 showed no evidence of κ gene rearrangement. Case numbers are shown above each lane. Adjacent dashes indicate the germline *Bam*HI restriction fragment size (12 kilobase, kb) hybridizing with the C_κ probe.

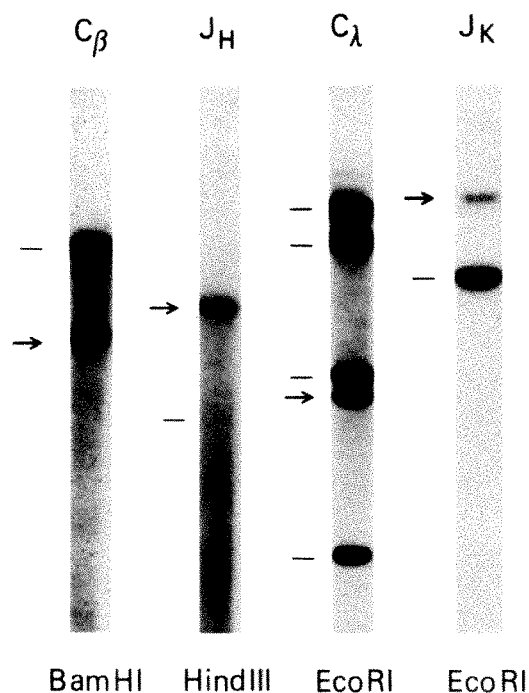


Fig 6. Gene rearrangements in the L428 Hodgkin's cell line. By Southern analysis, single gene rearrangements were detected for the β subunit of the T cell antigen receptor (C_β), immunoglobulin heavy chain (J_H), κ light chain (J_κ), and λ light chain (C_λ) (arrows). Location of germline restriction fragments (—).

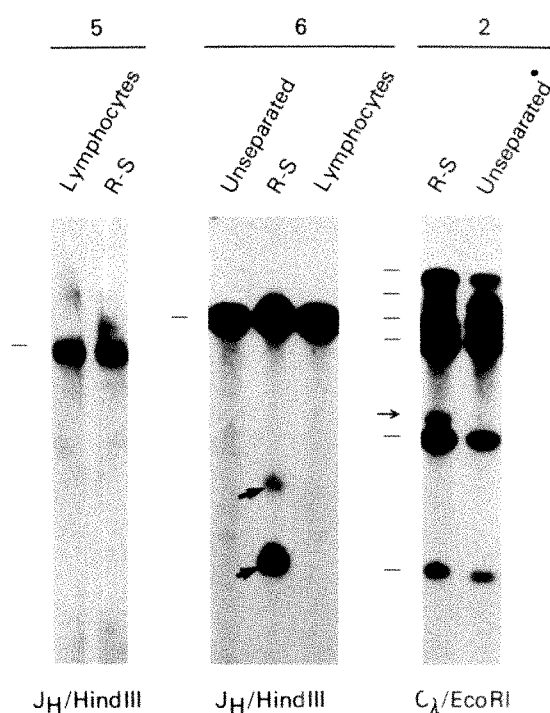


Fig 5. Immunoglobulin gene rearrangement in Reed-Sternberg cell-enriched fractions. Cell types were separated as outlined in Table 1, and DNA was extracted and analyzed for gene rearrangements in Southern blots. Case numbers are at top. In case 5, no rearrangements of the immunoglobulin heavy chain gene were detected in either the lymphocyte or Reed-Sternberg cell-enriched (RS) fractions. The RS fraction from case 6 contained two nongermline bands (arrows) hybridizing with J_H in *Hind*III-digested DNA. In contrast, no rearrangements were seen in either the unseparated cells or lymphocyte fractions from case 6. In case 2, the RS fraction exhibited a single rearrangement of the λ gene (arrow), whereas no λ rearrangements were detectable in unseparated cells; germline sizes (—).

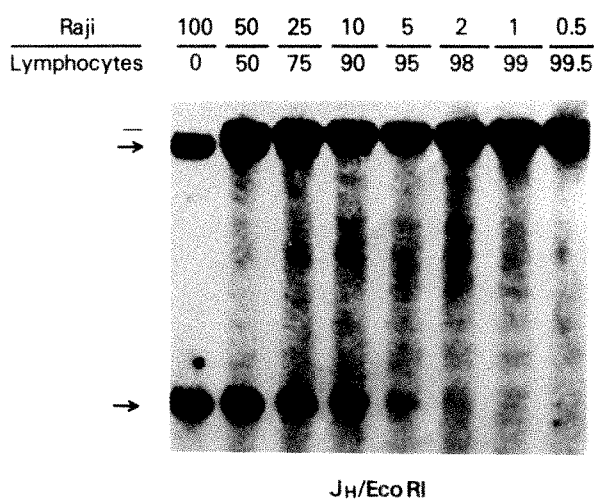


Fig 7. Sensitivity of Southern blot. Raji DNA was diluted in DNA obtained from normal polyclonal splenic lymphocytes. The percentages of total DNA contributed by either Raji or lymphocytes is indicated above each lane. DNA was digested with *Eco*RI and probed with J_H . Germline fragment (—); the two rearranged J_H loci in Raji cells (arrows).

INFORMATION FOR CONTRIBUTORS

SUBMITTING THE MANUSCRIPT

BLOOD, The Journal of The American Society of Hematology, provides an international forum for the publication of original articles, in English, describing basic laboratory and clinical investigations encompassed in the broad discipline of hematology. The scope of the journal covers all aspects of hematology, including disorders of leukocytes, both benign and malignant, erythrocytes, platelets, hemostatic mechanisms, and immunology, as well as major developments in clinical laboratory diagnosis and blood banking.

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The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which work was done; (4) acknowledgments for research support; (5) name and address of the author to whom communications regarding the manuscript should be directed.

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Personal communications and references to publications in press by authors other than those submitting the paper must be substantiated by a letter from the investigator(s) concerned confirming the data or observations and granting the authors permission to cite the material.

EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. *Br J Haematol* 38:161, 1978

Journal article, in press:

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Complete book:

3. Lillie RD: *Histopathologic Technic and Practical Histochemistry* (ed 3). New York, Blakiston, 1965, p 39

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4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): *Hemic Cells In Vitro*, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): *Gamma Globulins, Proceedings of the Third Nobel Symposium*. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Poliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. *Clin Res* 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sick cell anemia monthly variations. *Blood* 47:879, 1976 (letter)

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ANNOUNCEMENTS

Announcements of meetings, conferences, and the like that are of interest to the readership of *BLOOD* should be sent to the Editor at least three months before the first day of the month of issue. Fees for announcements vary, depending on their size and the number of issues involved. Prices may be obtained by contacting the *BLOOD* office.

DIRECTOR, TRANSFUSION SERVICE

The Department of Pathology, Rhode Island Hospital, seeks a Director for its Transfusion Service. The successful candidate must have demonstrated scholarly interests to qualify for a Brown University faculty appointment at the rank of Assistant or Associate Professor of Pathology. The candidate will be expected to administer a laboratory serving a 719-bed hospital population, participate in resident and medical student teaching, continue an established research interest or initiate/collaborate in a research program, preferably in the area of auto-antibodies to blood products and cells. Candidates with funded research and/or expertise in coagulation will be given preference.

Minimal requirements: M.D., certified in CP, BB-certified or qualified (American Board of Pathology), experienced in Transfusion Medicine, with administrative experience and research interests.

Deadline for receipt of applications: October 15, 1987. Send CV and names of 3 references to: Tito Cavallo, M.D., Chairman, Transfusion Service, Department of Pathology, Rhode Island Hospital, 593 Eddy St., Providence, RI 02902. EEO/AA employer.

CHIEF Bone Marrow Transplant Program

Roswell Park Memorial Institute is seeking candidates for the position of Chief of its Bone Marrow Transplant Program. This program will be a separate division within the Institute reporting to the Associate Director for Clinical Affairs and will be responsible for research in transplantation and the management of the Bone Marrow Transplant Unit. Roswell Park will serve as the regional center for transplants in oncology, aplastic anemia, combined immunodeficiency, and genetic disorders in Western New York. Roswell Park is affiliated with the State University at Buffalo School of Medicine.

The applicant should be an MD specialized in Internal Medicine or Pediatrics with special training in marrow transplantation and a record of productive individual and collaborative research.

Those interested should contact:

Dr. Edward Henderson
Department of Medical Oncology
Roswell Park Memorial Institute
666 Elm Street
Buffalo, NY 14263
(716) 845-3221

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Respond by letter stating the reason for your interest in a career in blood banking and a complete curriculum vitae immediately to:

Thomas F. Zuck, M.D.
Director
Hoxworth Blood Center
University of Cincinnati Medical Center
3231 Burnet Avenue
Cincinnati, Ohio 45267-0055

Chief of Hematology Laboratory

Full-time appointment as an Assistant Professor in Residence in the Department of Laboratory Medicine, University of California, San Francisco, at the San Francisco General Hospital. Minimum requirements include an M.D. degree, appropriate training and experience in hematopathology or hematology, board certification or eligibility, and displayed ability in teaching and research. Service responsibilities also include interpretation of bone marrow examinations and clinical consultation. Send curriculum vitae to: Jack Levin, M.D., Chairman, Hematology Search Committee, VA Medical Center (113A), 4150 Clement Street, San Francisco, CA 94121. UCSF is an Equal Opportunity/Affirmative Action Employer. Women and minorities are encouraged to apply.

BOARD REVIEW COURSE IN MEDICAL ONCOLOGY

**The George Washington University
Medical Center
Washington, DC**

October 14-18, 1987

The Division of Hematology and Oncology of the George Washington University Medical Center is pleased to announce this course to be held at the Omni Shoreham Hotel, October 14-18, 1987, in downtown Washington, DC.

Designed to meet the needs of physicians planning to take the medical oncology boards, this course will be a valuable update on the recent advances in oncology. It will satisfy the requirements for up to 37 hours of category I AMA credit. The course director is Philip Cohen, M.D.

For further information and registration, contact: Greg P. Thomas, Office of Continuing Medical Education, 2300 K Street, N.W., Washington, DC 20037; (202) 994-4285.

FACULTY POSITION IN DIVISION OF HEMATOLOGY/ONCOLOGY

Temple University School of Medicine needs a faculty member at the rank of Assistant Associate Professor level to join in the development of a new Bone Marrow Transplantation Program. Candidates' research and clinical efforts will be committed to Bone Marrow Transplantation Program. Candidates should send C.V. and letters of reference to:

Kenneth F. Mangan, MD

**Dept. of Bone Marrow Transplantation
Div. of Hematology/Oncology**

9th Floor

Parkinson Pavilion, Rm. 904

**Broad & Tioga Streets.
Philadelphia, PA 19140**

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Bone Marrow Transplantation Program Director

Marshfield Clinic is seeking a Director for bone marrow transplantation programs. The Clinic is a 250 physician, multispecialty group practice. The Department of Clinical Oncology is composed of 5 Oncologists, 3 Hematologists, 2 Hematologist/Oncologists and 2 Radiation Oncologists. We are a CCOP and active member of ECOG, NSABP, RTOG and CCSG. An active autologous transplantation program exists and several department members are experienced in allogeneic transplantation. Clinical research opportunities are readily available and basic research facilities through the Marshfield Medical Research Foundation also exist. Responsibility of the Physician Director will be to develop a comprehensive bone marrow transplantation program for both adult and pediatric patients. Interested candidates should submit a curriculum vitae or contact:

William G. Hocking, M.D.
Department of Clinical Oncology
(715) 387-5426

1000 North Oak Avenue
Marshfield, WI 54449



Marshfield Clinic

Immunohematology Fellowships

The American Red Cross Blood Services offers accredited postdoctoral fellowship training in immunohematology and transfusion medicine for qualified candidates in several of its regions, including:

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LOS ANGELES-ORANGE COUNTIES REGION
LOS ANGELES, CALIFORNIA

NORTHWEST OHIO REGION
TOLEDO, OHIO

PENN-JERSEY REGION
PHILADELPHIA, PENNSYLVANIA

SOUTHEASTERN MICHIGAN REGION
DETROIT, MICHIGAN

SOUTH FLORIDA REGION
MIAMI, FLORIDA

ST. PAUL REGION
ST. PAUL, MINNESOTA

These programs offer didactic training, practical experience and clinical responsibilities in blood centers and affiliate medical centers. For further information, contact:

James P. AuBuchon, MD
Medical Officer
American Red Cross
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1730 E Street NW
Washington, DC 20006



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